

PATENT

UTSC:305

COMPOSITIONS AND METHODS FOR ELICITING  
IMMUNE OR ANTI-INFECTIVE RESPONSES

By:

Jagannadha K. Sastry,

Ralph B. Arlinghaus,

Chris D. Platsoucas,

and

Pramod N. Nehete

"Express Mail" mailing label

number EM423825112US

Date of Deposit JUNE 5, 1997

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231

JOHN MCDAVITT

Typed or printed name of person mailing paper or (fee)

[Signature]  
Signature of person mailing paper or (fee)

"Express Mail" mailing label

number RB 873 744 729 US

Date of Deposit September 16, 1992

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Onita Cheyne

(Typed or printed name of person mailing paper or fee)

[Signature]  
(Signature of person mailing paper or fee)

**BACKGROUND OF THE INVENTION**

The government may own certain rights in the present invention pursuant to NIH grant AI-29308.

10 This is a continuation-in-part of co-pending application  
Serial No. 07/800,832, filed December 2, 1991, which was a  
continuation-in-part of Serial No. 07/410,727, filed September  
20, 1989, now issued as U.S. Patent 5,128,319, which in turn was  
a continuation-in-part of Serial No. 07/090,646 filed 28 August,  
15 1987.

**1. Field of the Invention**

20 The present invention relates generally to the prevention  
and treatment of viral infections. More particularly, this  
invention concerns the identification of distinct classes of  
peptides which may be advantageously combined for use in both  
anti-viral vaccines and therapeutic formulations. Peptide  
formulations are disclosed which enhance the systemic  
25 distribution, activity, and longevity of anti-viral cytotoxic  
T cells, and/or which protect human cells from HIV infection.

**2. Description of the Related Art**

30 AIDS was first recognized in the United States in 1981; the  
number of cases has been increasing at a dramatic pace since  
then. Since 1978 more than 2.4 million AIDS infections have been  
reported in the United States, alone (Rees, 1987). Once  
significant immunosuppressive symptoms appear in an infected  
35 individual, the expected outcome of the infection is death.  
There is currently no known treatment that can indefinitely delay  
or prevent the fatal consequences of the disease. Although the  
disease first manifested itself in homosexual or bisexual males  
and intravenous drug abusers, it has now spread to others by

means such as intimate sexual contact with or receipt of blood products from a carrier of the virus.

5 The causative agent, associated with AIDS has been identified as a group of closely related retroviruses commonly known as Human T Cell Lymphotropic Virus-type III (HTLV-III), Lymphadenopathy Viruses (LAV), AIDS-Related Viruses (ARV), or more recently named Human Immunodeficiency Virus (HIV). These viruses will be collectively referred to herein for convenience as HIV.

15 Like other retroviruses, HIV has RNA as its genetic material. When the virus enters the host cell, a viral enzyme known as reverse transcriptase copies the viral RNA into a double stranded DNA. The viral DNA migrates to the nucleus of the cell where it serves as a template for additional copies of viral RNA which can then be assembled into new viral particles. The viral RNA can also serve as messenger RNA (mRNA) for certain viral proteins, including the viral core proteins p18, p24 p13, and reverse transcriptase. RNA may also ~~be~~ be "spliced" into specific viral mRNAs necessary to produce several other viral proteins including two glycosylated structural proteins known as gp41 and gp120 which are inserted in the outer membrane of the virus (Wain-Hobson et al., 1985). Purified gp120 is known to induce antibody in the goat, horse and rhesus monkey that neutralizes HIV in lab tests (Robey et al., 1986).

30 Vaccines have been used for many years to prevent infections caused by agents such as viruses. The general approach has been to inject healthy individuals with, for example, a killed or modified virus preparation in order to prime the individual's immune systems to mount an assault on the infecting virus. Recent advances in recombinant DNA technology have allowed safer methods of vaccination that involve use of exposed viral components produced by microbial systems. After sufficient

purification, the viral component, for example a protein subunit, is administered as a vaccine in a suitable vehicle and/or an adjuvant. The latter stimulates the host's system in a way that improves the immune response to the viral subunit.

5

Another potential method of making a vaccine is by using chemically synthesized peptide fragments of a viral protein subunit. This method has several advantages over the other methods of producing vaccines, including purity of the product, reproducibility and specificity of the immune response.

10

Surface antigens of an infecting virus can elicit T cell and B cell responses. From the work of Milich and coworkers (Milich et al., 1986; Milich & McLachlan, 1986) it is clear that some regions of a protein's peptide chain can possess either T cell or B cell epitopes. These epitopes are frequently distinct from each other and can comprise different peptide sequences. Other examples include the work of Maizel et al., (1980) for hen egg-white lysozyme, and Senyk et al., (1971) for glucagon. Thus, short stretches of a protein sequence can elicit a T cell response but not a B cell response. A more complete review of these and other observations pertinent to this point is included in the work of Livingstone & Fathman (1987).

15

20

25

A short peptide region within the surface protein of infectious Hepatitis B virus has been shown to elicit only a T cell response in mice (Milich et al., 1986). Specifically, a synthetic peptide, whose sequence is derived from amino acids numbered 120-132 located within the pre-S(2) domain of the Hepatitis B surface antigen gene, elicited a very strong T cell priming response to the peptide but stimulated only a very weak antibody response. In other words, mice mounted a poor antibody response to that peptide, but the T cells of immunized mice were efficiently primed (i.e. activated) to recognize that peptide as measured in T cell proliferation assays (Milich et al., 1986).

30

35

The low level of the antibody produced by mice immunized with this peptide did not bind to the native viral surface antigen.

In contrast to the above-described results, a second peptide sequence (amino acids 132-145) elicited a very weak T-cell response in mice (Milich et al., 1986). This second peptide did, however, efficiently bind antibody raised against it under conditions where a T cell epitope is provided.

Mice were also immunized with a longer peptide made up of both of the above-mentioned T- and B-active peptide sequences. In this case, high titers of antibody were produced against the B site peptide but not the T site peptide. The combination of both T- and B-sites within one peptide should stimulate both T and B cell responses, as measured by producing a specific antibody to the B cell epitope of the peptide chain. Synthetic peptide antigens may be constructed to produce two types of immune responses: T-cell only and T cell combined with a B cell response.

Cellular immune responses provide a major mechanism for reducing the growth of virus-infected cells (Doherty et al., 1985). A report by Earl et al., (1986) demonstrated T-lymphocyte priming and protection against the Friend virus (a retrovirus)-induced mouse leukemia by a viral surface protein vaccine. Direct evidence for the role of a subset of T-lymphocytes (OKT8/LEU2 positive) in suppressing HIV growth *in vitro* has been obtained by Walker et al. (1986). This study further demonstrated that, after depletion of CD8<sup>+</sup> T-lymphocytes from the blood of HIV-infected individuals, large quantities of HIV were isolated from peripheral blood mononuclear cells of four of seven asymptomatic, seropositive homosexual men who were initially virus-negative or had very low levels of virus. Thus, the CD8<sup>+</sup> cytotoxic T-lymphocytes (CTLs) may play a role in virus infected individuals to prevent HIV replication and disease progression.

5 The concept of identifying T-cell epitopes in proteins for  
inclusion in potential vaccine candidates has gained importance  
as a result of the demonstration by Townsend et al. (1986) that  
CTL epitopes of influenza nucleoprotein can be defined by short  
synthetic peptides. However, to date there are only three  
documented cases (Deres et al., 1989; Aichele et al., 1990; Kast  
et al., 1991) that describe the use of synthetic peptides in the  
in vivo priming of CTLs, these relate to influenza, Sendai and  
lymphocyte choriomeningitis viruses. In each of the above cases,  
10 the immunization protocols are cumbersome, require either  
modifications of peptides or many immunizations to be carried out  
to demonstrate CTLs, and do not lend themselves to the rapid  
screening of a large number of candidate substances. For  
example, the method of Aichele and colleagues (1990) involves  
15 three immunizations at one week intervals by the subcutaneous  
route, and takes four weeks before potential CTLs are obtained  
for assaying.

20 Candidate CTL epitopes in both structural and regulatory HIV  
proteins have been proposed (Takahashi et al., 1988; Nixon et  
al., 1988) but none of these have been shown to be capable of  
inducing virus-specific CTLs in vivo (Berzofsky, 1991). For  
example, although the peptide RIQRGPGRAFVTIGK (R15K) has been  
identified as a CTL epitope (Takahashi et al., 1988), in these  
25 studies the in vivo induction of R15K-specific CTLs was  
accomplished by infecting Balb/c mice with recombinant vaccinia  
virus expressing HIV env proteins (Takahashi et al., 1988) and  
attempts at immunization with free peptide have been unsuccessful  
(Berzofsky, J.A., 1991). Hart et al. (1991) were also unable to  
30 generate CTL responses on immunization with a single peptide  
having the CTL epitope sequence, CTRPNNNTRKSIRIQRGPGRAFVTI.

The mechanisms underlying the induction of peptide-induced  
CTL responses in vivo are not yet fully understood. For  
35 instance, Gao et al (1991) reported that the addition of a

T helper determinant to a CTL determinant, to create a hybrid peptide, did not enhance CTL generation against influenza virus. These results suggest that the induction of T helper cell activity is not specifically required for effective CTL generation, although the same studies showed that depletion of CD<sup>+</sup> cells inhibited CTL generation in response to peptides.

Thus, there remains a need for both the development of techniques for the rapid identification of CTL epitopes that have the ability to induce a specific CTL response *in vivo*, and for the optimization of CTL induction. Previous assay methods for the identification of CTL-inducing epitopes that will function *in vivo* have suffered a number of drawbacks. The most notable of which are: the requirement for multiple injections of the material to be tested, a wait of up to 3 weeks or longer to determine whether the substance had a positive effect on CTL response, and the general need to include a modifier with the substance being tested in order to elicit a response. Clearly, a rapid method for the delineation of peptides with an *in vivo* CTL inducing capacity is of vital importance in the design of preventative and therapeutic strategies in relation to a wide variety of diseases.

The art currently also lacks an effective method of producing a systemic, long-lived and high level CTL response following peptide immunization. The development of a method to enhance the generation and systemic distribution of anti-viral CTL cells, in animals or humans, would be of great advantage in the development of vaccines against infectious viral agents. Not only would such a method be an important weapon for use against viruses, including AIDS and influenza, but it would also be applicable to vaccination against other agents such as parasites.

Although (it) the gp120 V3 loop region is known to be essential for HIV-1 entry into cells (Travis et al., 1991; Freed

& Riser, 1987; Freed et al., 1991), this knowledge has yet to lead to the development of an effective clinical strategy to prevent HIV infection. V3-derived synthetic peptides have been reported to inhibit syncytium formation between the HIV-1 infected cells, but only at concentrations of 100-300  $\mu$ M (Koito et al., 1989). In contrast, De Rossi et al. (1991) reported that V3-derived synthetic peptides actually enhanced HIV-1 infection of cells through a CD4-dependent mechanism. Therefore, in addition to the distinct lack of a suitable vaccine against HIV, there are also currently no effective means for arresting viral infection and for preventing disease progression in HIV-infected individuals.

#### SUMMARY OF THE INVENTION

The present invention addresses one or more of the foregoing or other disadvantages in the prior art by providing, firstly, compositions and methods for immunization against viral diseases, and secondly, therapeutic compositions and methods for use in inhibiting HIV infection. The improved immunization compositions, or vaccines, of the invention comprise synthetic peptide combinations which enhance the systemic distribution, level of activity, and longevity of virus-specific cytotoxic T cells (CTLs). The therapeutic formulations of the invention comprise synthetic peptides which function to protect human cells from HIV infection. The present invention therefore widely encompasses anti-viral, and particularly anti-HIV, compositions which have both preventative and therapeutic applications.

In important embodiments, the present invention concerns compositions and methods for enhancing the CTL response of an animal to a given immunogen, and particularly to a CTL-inducing peptide. A CTL-inducing peptide is a peptide, bearing a CTL-inducing epitope, which is capable of stimulating the formation,



or increasing the activity, of specific cytotoxic T cells following its administration to an animal. The term "enhancing the CTL response", is used herein to encompass improvements in all aspects of the CTL response, such as for example, improving the systemic distribution, level of activity, and longevity of the response to CTL-inducing peptides.

The method of the present invention for enhancing a CTL response to an immunogen, such as a peptide, involves the addition of a composition including a separate and distinct class of peptides to the immunization mixture that possess T helper cell-inducing activity. To achieve enhancement of the CTL response in this manner one would therefore administer to the animal, in addition to the immunogen itself, an immunologically effective amount of a peptide bearing a T helper cell epitope.

In a related embodiment, the present invention provides a method of identifying a candidate substance capable of enhancing a CTL response. To identify such an enhancing substance, such as a peptide, one would administer to an animal both the candidate substance and an immunogen capable of inducing a CTL response. The CTLs would then be recovered from the animal and their activity, including systemic distribution and longevity, would be determined. A candidate substance capable of enhancing a CTL response would be identified as a substance that increased the CTL response, using any of the above parameters, over that observed in the presence of the immunogen alone.

The immunization methods of the present invention are generally applicable to enhancing CTL responses towards immunogens from any source, including those from a wide variety of infectious agents such as viruses or parasites. However, in preferred embodiments, the invention is exemplified by enhancing the CTL response against components of the HIV viral family. CTL responses may be generated against epitopes located within the

products of any viral gene, such as, for example, the *gag*, *pol*, *nef* and *env* genes, with the products of the *env* genes being preferred targets.

5           Any one of a wide variety of peptide sequences may be employed as a CTL-inducing epitope in accordance with the present invention. These include the peptides listed in Table 1, which is, of course, an exemplary and not exhaustive list. In preferred embodiments, it is contemplated that peptides from the  
10 *env* (envelope) gene product, and more preferably, those derived from the V3 loop of HIV gp120, will be employed as CTL-inducing peptides. An exemplary list of V3 loop peptides from a variety of HIV-1 isolates is provided in Table 2. A specific example of a V3 loop-derived peptide found to be successful in generating  
15 CTL responses is the peptide R15K, having the sequence RIQRGPGRAFVTIGK (seq id no:1).

1-7 //

Table 2

Seq id no:1  
RIQRGPGRAFVTIGK  
2010-01-10

TABLE 1  
CANDIDATE ANTI-HIV CTL-INDUCING EPITOPES

	GENE	PEPTIDE	SOURCE OF CTLs	MHC/HLA	REFERENCE
5	gag	K25E 18-42	Human	Bw62	Johnson et al., 1991
		Q25E 69-93	Human	A2	"
		V22F 148-164	Human	Bw57	"
		N22A 153-174	Human	Bw57	"
		S22H 173-194	Human	B14	"
		G22R 193-214	Human	Bw52	"
10		N22V 263-274	Human	De	"
		Y19T 262-280	Mouse	H-2d	Michel et al., 1992
		K22D 263-284	Human	Bw62	"
		K16C 285-279	Human	B27	Nixon et al., 1988
		R10K 305-310	Human	B14	Johnson et al., 1991
15	pol	I25E 172-196	Human	B8	Walker et al., 1989
		C17P 205-219	Mouse/Human	H-2k	Hosmalin et al., 1990
		A25Q 325-349	Human	A11	Walker et al., 1989
		N26R 348-366	Human	A11	"
20		D25T 359-383	Human	Bw60	"
		P25Y 461-485	Human	Bw60	"
		E25G 495-519	Human	A11	"
25	env	L22A 25-46	Human	A2	Dadaglio et al., 1991
		T20K 196-212	Human	A2	"
		S17I 295-311	Human	A2	"
		R15K 315-329	Mouse/Human	H-2d/A2	Berzofsky et al., 1991
		P7S 374-380	Human	A2	Dadaglio et al., 1991
		K12S 381-392	Human	A2	"
30		L20Y 421-440	Human	A2	"
		Y 101-610	Human	A1	
		E9L 584-592	Human	B14	Johnson et al., 1992
		Y8L 586-593	Human	B8	"
35	nef	Q25L 73-97	Human	A3.1	Koenig et al., 1990
		W10T 110-120	Human	B17B37	Culmann et al., 1989
		D16A 176-191	Mouse	H-2d	Michel et al., 1992
		E17L 184-199	Mouse	H-2d	"

TABLE 2

## SYNTHETIC PEPTIDES FROM THE V3 LOOP OF VARIOUS HIV-1 ISOLATES

PEPTIDE NO.	STRAIN	SEQUENCE
D23 (24aa)	MN	YNKRKRIHIGPGRAFYTTKNNIGC. 16
D24 (15aa)	MN	RIHIGPGRAFYTTKN 12
D25 (15aa)	WMJ-3	SLSIGPGRAPRTREI 12
D26 (24aa)	RF	NNTRKSITKGPGRVVIYATGQIIGD 19
D27 (15aa)	NY-5	GIAIGPGRTLYAREK 5
D28 (15aa)	RF	SITKGPGRVVIYATGQ 16
D29 (15aa)	CDC4	RVTLGPGRVWYTTGE 7
D30 (15aa)	SC	SIHIGPGRAFYATGD 8
D31 (15aa)	Z3	SIRIGPGKVFTAKGG 9
D32 (15aa)	SF2	SIYIGPGRAFHTTGR 20
D33 (15aa)	MAL	GIHFGPGQALYTTGI 1
D34 (15aa)	Z321	SISIGPGRAFFATTD 2
D35 (15aa)	Z6	STPIGLGQALYTTRG 3
D37 (15aa)	JY1	STPIGLGQALYTTRI 11
D38 (15aa)	ELI	RTPTGLGQSLYTTRS 5
D39 (15aa)	MN (Y-L)	RIHIGPGARFLTTKN 6
D40 (15aa)	MN (Y-F)	RIHIGPGRAFFTTKN 7
D44 (15aa) (R15K)	IIIB	RIQRGPGRAFTIGK

Various methods are available to identify T helper cell-inducing epitopes suitable for use in accordance herewith. For example, the amphipathicity of a peptide sequence is known to effect its ability to function as a T helper cell inducer. In preferred embodiments, it is contemplated that one would wish to employ a T helper cell-inducing epitope having an amphipathicity value of from about plus 10 to about plus 20. Other quantifiable characteristics include, for example, having an alpha helix turn of  $100 \pm 15$  degrees, or a  $3_{10}$  helix turn with  $120 \pm 15$  degrees. In embodiments relating specifically to HIV, T helper cell-inducing peptides including sequences derived from an HIV gp120 sequence which have an amphipathicity value within the above range will be preferred.

Again in relation to HIV, the use of peptides containing the sequence CRIKQIINMWQGVGKAMYA (C19A, seq id no:2) is preferred as this peptide was found to be particularly effective at enhancing CTL responses. However, peptides including other T helper cell-inducing epitopes may be employed. A full discussion of T helper cell-inducing epitopes is given in U.S. Patent 5,128,319, incorporated herein by reference.

Related embodiments of this invention concern compositions for effecting improved CTL responses. Such compositions will generally include at least two peptides, the first of which will contain the CTL-inducing epitope against which the immune response is specifically desired, and second of which will comprise a T helper cell-inducing epitope. Naturally, a combination of such a composition in which the peptides are dispersed in a pharmacologically acceptable vehicle would be an ideal formulation for use as a vaccine.

The present invention further relates to the identification of synthetic peptides which protect human cells from HIV-1 infection. Such inhibitory peptides may be employed to inhibit

HIV infection of cells, for use, for example, in assay protocols and as therapeutic agents for use in the treatment of AIDS.

As used herein, the term "HIV infection-inhibiting sequence" refers to a peptide sequence which prevents entry of the HIV virus into its target cell. As such, an inhibitory peptide may be characterized as including a peptide sequence that is involved in the infection process, or that functions to contact the target cell. Infection-inhibiting peptides particularly include peptides that comprise a sequence wherein antibodies against that sequence are capable of inhibiting HIV cellular infection.

The present invention discloses that synthetic peptides with sequences derived from the HIV-1 env gene product, gp120, have the capacity to inhibit HIV cellular infection. In particular, the inventors have identified HIV infection-inhibiting sequences within the V3 loop and at the N-terminal regions of gp120. It is also contemplated that HIV infection-inhibiting sequences may prove to be located within the CD4 binding region.

In preferred embodiments, the inventors contemplate the use of HIV infection-inhibiting peptides with sequences derived from the gp120 V3 loop. As detailed herein, peptides found to be of use in this regard include, for example, those peptides of Table 11, and particularly, peptides D23, D24, D25, D26, D30, D35, D38, D39, D40 and D44 (R15K), the sequences of which are shown in Table 11A. These peptides have sequences derived from the V3 loops of a variety of strains such as mn, rf, wmj-3, sc, z6, eli, mn (y-l) and mn (y-p). Preferred V3-derived infection-inhibiting peptides are contemplated to include those listed in Table 11A, and those such as R15K, having the sequence RIQRGPGRAFTIGK (seq id no:1), and also N24G, having the sequence NNTRKSIRIQRGPGRAFTIGKIG (seq id no:3).

An important aspect of the present invention is the

"D23, D24, (SK)" and insert here?

discovery that peptides with sequences that correspond to sequences from a variety of different HIV isolates have the ability to inhibit HIV infection of cells. Further to those shown in Table 11A, these peptides include, for example, H13N (HIGPGRAFYT<sup>2</sup>TKN, seq id no:7), a V3-loop peptide from HIV-1mn strain, and T13Q (TKGPGRVIYATGQ, seq id no:6), a V3-loop peptide from HIV-1rf strain. However, in regard to Table 11, it is important to note that this data was compiled from assays specifically directed to inhibiting infection by one HIV strain, namely HIV-1 IIIB. Therefore peptides such as (D27, D28, D29, D31, D32, D33, D34 AND D37, listed Table 11B,) which may not show activity in one specific inhibitory assay may still have utility in preventing the infection of target cells by a variety of other HIV strains, for example against HIV strains ny-5, rf, cdc4, z3, sf2, mal, z321 and jy1.

The inventors have also identified peptides from the gp120 N-terminus, such as E13V, having the sequence EQLWVTVYYGVPV (seq id no:4), as infection-inhibiting peptides. This is an important finding as this area of gp120 is known to be relatively conserved between different HIV strains.

It should also be noted that smaller peptides which include portions of these sequences, such as, for example, R8K, having the sequence RAFVTIGK (seq id no:5), have also been identified as having infection-inhibiting sequences. As such, smaller peptides and peptide fragments are considered to be useful in accordance herewith and to be within the scope of this invention.

The infection-inhibiting peptides of the present invention may include natural, or engineered, sequence variations and yet still function to prevent HIV entry into cells. As such, the biological functional equivalents of the peptides in Tables 1, 2 and 11, and peptides such as R15K, N24G, E13V, H13N and T13Q, are also considered to fall within the scope of the present

invention.

Such biological functional peptide equivalents may include changes based upon hydropathic index of amino acids. It is believed that the relative hydropathic character of the amino acid determines the secondary structure of the resultant protein or peptide, which in turn defines its interaction with other molecules, such as for example, receptors, antibodies, and the like. It is generally known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still result in a peptide with similar biological activity (Kyte & Doolittle, 1982).

The hydropathic indices of the amino acids are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). Biological functional equivalents are considered to be those peptides which include the substitution of amino acids whose hydropathic indices are within  $\pm 2$ , and more preferably, within  $\pm 1$ , and even more preferably, within  $\pm 0.5$ .

Substitution of like amino acids can also be made on the basis of hydrophilicity, particularly where the biological functional equivalent protein or peptide thereby created is intended for use in immunological embodiments, as is the case for the present invention. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e. with a biological property of the protein.



As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5  $\pm$  1); threonine (-0.4); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). Similarly, biological functional equivalents are considered to be those peptides which include the substitution of amino acids whose hydrophilicity values are within  $\pm$ 2, and more preferably, within  $\pm$ 1, and even more preferably, within  $\pm$ 0.5.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition to the specific sequences described above, and their biological functional equivalents, other sequences may be employed as HIV infection-inhibiting peptides. For example, as mentioned above, it is contemplated that any sequence where anti-peptide antibodies against that sequence have been shown to inhibit viral entry into cells will be a suitable HIV infection-inhibiting peptide for use in accordance herewith. The advantages of the present invention include the demonstration that such peptides can function effectively at low concentrations, such as from between about 1  $\mu$ g/ml to about 1 ng/ml.

In any event, in light of the present disclosure, candidate sequences with the potential to function as HIV infection-inhibiting peptides can now be identified and tested. Such candidate peptides will likely include sequences located within the gp120 V3 loop or N-terminal region, or even within the CD4 binding region of gp120, and preferably, will be sequences against which antibodies have been raised and have been shown to inhibit or reduce HIV cellular infection. Candidate sequences may be synthesized as peptides and tested for the ability to inhibit HIV cellular infection.

As discussed above, in the compositions of the present invention, peptide(s) containing CTL-inducing epitope(s) may be combined with peptide(s) including T helper cell-inducing epitope(s) to create immunization, or vaccine, formulations. In regard to peptide cocktails for immunization, the use of peptides with sequences which correspond to the more conserved areas of HIV gp120 is generally preferred. Additionally, and specifically relating to HIV, the CTL-inducing peptide(s) may be combined with HIV infection-inhibiting sequence(s) as therapeutic formulations.

However, and importantly, compositions comprising all three of the distinct classes of peptide identified herein are also contemplated by the present invention. Such a single formulation would be capable of preventing infection of human cells by HIV-1 and of inducing HIV-1 specific CTL responses. Since all of these formulations are completely synthetic and defined, these reagents will be both economical and safe.

Naturally, any one of the peptides may have more than one immunological activity. This phenomenon is exemplified by the peptide R15K which has CTL-inducing and infection-inhibiting properties. In the most preferred embodiments, it is contemplated that peptides which do not induce the significant production of antibodies that bind to native HIV will be

employed. In addition to being used directly as monomers, the peptides of the present invention may also be formulated as polymers or lipid-tailed peptides.

5 In further embodiments, the present invention concerns methods for assaying a composition for its ability to induce a cytotoxic T cell (CTL) response in an animal. Related to this, are methods for assaying a composition for its ability to enhance a CTL response induced by a distinct component. These screening  
10 methods include generally immunizing an animal, such as a human subject or an experimental animal such as a mouse, rat, rabbit, guinea pig, goat, rhesus monkey, or chimpanzee, thereafter collecting cells from lymph nodes or other lymphoid tissue from the animal, and then testing the tissue for the presence of CTLs that are primed to kill or lyse cells producing a component of an  
15 infectious agent, such as a viral envelope, core protein, one of the functional proteins (e.g., reverse transcriptase) or the like.

20 Thus, the method of the invention includes generally three steps, with the first step involving immunizing an animal with the composition to be tested. While it is believed that any accepted mode and route of immunization can be employed and nevertheless achieve some advantages in accordance herewith, the  
25 inventors have found particular advantages to be associated with intradermal immunization. Intradermal immunization is believed to be preferred because it serves to activate more efficiently the cell mediated arm of the immune system. Moreover, although where desired one may choose multiple injections of the  
30 immunizing substance, and even multiple sites of injection, the inventors have found that a particular advantage of the invention is that a single injection of the candidate will usually be sufficient to achieve detectable CTL activation in a nearby lymph node. Furthermore, although where desired one may employ  
35 immunization modifiers such as T-helper cell peptide sequences,

lipid structures that induce micelle formation, peptide polymerization methods, or the like in association with the candidate composition, the inventors have found that the sensitivity of the assay is such that the use of such modifiers will generally not be required in order to achieve CTL priming.

Once immunization has been effected, it will then be necessary to recover cytotoxic T cells from lymphoid tissue of the immunized animal. The preferred lymphoid tissue will be lymph node tissue, and most preferably tissue from draining lymph nodes proximal to the site of injection. As used herein, the word "proximal node" is intended to refer to the node or nodes that are located proximal to the site of injection, e.g. the popliteal node of the mouse following foot pad injection. It is believed that the use of proximal or draining nodal tissue to identify CTL activation is one reason for the rapidity of the more preferred aspects of the invention. Such nodes are physically located in the proximity of the immunization site, or in the area draining the site of immunization, and also included are those draining nodes that are physically at a greater distance from the immunization site.

The final step of the assay in its most general sense involves determining whether said cytotoxic T cells have been activated by the composition. Although it is not generally required, it will typically be preferred to actually measure the level of activation, through, e.g., radioactive chromium-release assays, or other radioisotope assays, or single cell assays; also single cell cytotoxic assays using vital stains and/or cell-sorters could be employed.

Where measurement of activation of cytotoxic T cells is desired, a preferred method involves contacting a killing effective amount of said cytotoxic T cells with MHC-matched target cells that exhibit the candidate epitope on their cell

surfaces; maintaining said contact for a time period sufficient for said cytotoxic T cells to lyse said target cells; and determining the degree of T cell-mediated lysis of said target cells. However, any method capable of detecting a specific CTL response may be employed, including but not limited to chromium-release assays, single-cell assays or even determination of cell-cell conjugates.

An advantage of the present invention is the speed with which one is able to determine the ability of the candidate substance to activate CTLs. Prior techniques have generally required a wait of a few weeks (Kast et al., 1991; Aichele et al., 1990). The present technique, though, typically requires only about 7 to 10 days following immunization. The reason for the reduced time necessary to achieve a CTL response with the assay of the present invention is believed to be the result of the route of immunization and the use of draining or proximal lymph node cells. A further advantage is the ability to test peptide candidates without the use of an associated modifier, such as carrier molecules, lipid tails, or T helper epitopes, to enhance its CTL activity.

Typically, the composition to be tested for CTL priming capability will comprise one or more peptides, or peptide multimers, believed to have or suspected of having useful activities. Through the application of the techniques of the present invention to such peptides, one will thus be enabled to determine whether such peptides do in fact have CTL priming activity. If so, then the peptide will be a candidate for inclusion in a CTL priming vaccine. It will be appreciated by those of skill in the art that while candidates for CTL priming activity will generally comprise peptides (or proteins), the use of the assay of the present invention in the context of non-peptidyl compounds is certainly not excluded.

It is proposed that the method of the present invention will find a broad range of application, particularly in the identification of components for use in the preparation of vaccines for the treatment and/or prevention of viral diseases such as AIDS, influenza, feline leukemia, bovine leukemia, Herpes virus infections, and even in the case of non-viral infectious diseases such as parasitic and bacterial infections. Therefore, in the case of embodiments directed to the identification of epitopes for promoting a specific anti-HIV CTL response, the invention will generally be concerned with the identification of peptides having the ability to direct a CTL response to HIV-infected cells.

In the context of vaccine development, the method will include first identifying a CTL-reactive composition in accordance with the foregoing method, and admixing the composition with one or more pharmaceutically acceptable diluents or additives, such as water, salts, emulsifiers and/or adjuvants. Of course, the amount of the composition added to the vaccine will vary depending on its ability to induce a specific CTL response, its solubility, and other biological responses. The selection of an appropriate amount of the identified CTL-priming composition will therefore be well within the skill of the art in light of the present disclosure.

In other embodiments, the invention involves a method of preparing cytotoxic T cells. In its most general sense, this method includes immunizing, preferably intradermally, an animal with a composition having the ability to induce a cytotoxic T cell response to a preselected epitope of a targeted protein. The epitope or epitopes employed may or may not be specific for CTL priming, and thus may or may not substantially induce antibodies that will cross react with the targeted protein. However, in the practice of this aspect of the invention, one will typically desire to recover cytotoxic T cells from lymph

nodes of the animal for further use. Numerous potential uses of specifically primed CTLs are envisioned. For example, in the case of human therapy, it is contemplated that specifically primed CTLs may be cultured and administered to humans for the treatment of viral infections or patients with cancer. In this case, the CTLs are prepared by immunizing the species *in vivo* and isolating the immune cells to expand *in vitro* in the presence of appropriate peptide, cytokines and presenting cells.

Typically, for the induction of an HIV-directed CTL response, the invention will involve the use of peptides which comprise from 7 to about 30 amino acid residues, and have a sequence that corresponds to a domain of an HIV protein such as the gp160 envelope and core proteins, reverse transcriptase, tat, rev or other gene products expressed by the virus, which peptide includes within its structure a conserved region.

For the preparation of vaccines, peptide multimers are generally preferred in order to include multiple CTL epitopes within a single complex. Two specific classes of peptide multimers are disclosed. In one class, the amino-terminal residue of a peptide is peptide-bonded to a spacer peptide that contains an amino-terminal lysyl residue and one to about five amino acid residues such as glycyl residues to form a composite polypeptide. Those added residues of the spacer peptide do not interfere with the immunizing capacity of the multimer, nor with its capacity to form surfactant-like micelles in aqueous compositions. The alpha- and epsilon-amino groups of the amino-terminal lysyl residue are amidified with a C<sub>12</sub>-C<sub>18</sub> fatty acid such as palmitic acid to form the reaction product that is used. The di-amide so formed forms surfactant-like micellular multimers in an aqueous composition.

A second class of multimer is a polymer having a peptide as a repeating unit. Here, each peptide is synthesized to contain a

cysteine (Cys) residue at each of its amino- and carboxy-termini. The resulting di-cysteine-terminated (di-Cys) peptide is then oxidized to polymerize the di-Cys peptide monomers into a polymer or cyclic peptide multimers in which the peptide repeating units are linked by cystine (oxidized cysteine) residues.

A peptide multimer of either class can contain one or a plurality of different peptide sequences. A peptide of a multimer is an "active" peptide in that when used in a composition discussed below, the multimer can induce cell mediated immunity such as production of cytotoxic T cells. A multimer can also include an inactive peptide, for example to assist in dispersing the multimer in the aqueous medium. The lysyl-containing peptide spacer discussed before can be viewed as such an inactive peptide.

The peptide multimer is utilized in an aqueous composition (inoculum). That composition contains water having a before-described multimer dispersed therein. The composition, when used to immunize an immunocompetent host animal such as a mouse, has the capacity of inducing cell mediated immunity such as cytotoxic T cell activation to the native HIV protein corresponding in sequence to that of an active peptide of the multimer, but does not substantially induce production of antibodies that immunoreact with that corresponding native HIV protein. The composition thus contains an immunizing effective amount of a before-discussed multimeric peptide.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. CTL activity of popliteal lymph node (PLN) cells from Balb/c mice after immunization with HIV env V3-loop R15K peptide (aa 315-329, seq id no:1) in three forms. A, linear monomer; B, di-sulphide-linked polymers formed by cysteine



residues added at both the N- and C- termini; C, lipid-tailed micelles formed by conjugation to a dipalmityl-lysine-glycine-glycine at the N-terminus (Hopp, 1984; Sastry et al., 1991). Balb/c mice (6-8 weeks of age) were immunized in the hind-foot pad with the peptide (100 $\mu$ g in a 1:1 emulsion with CFA). After 10 days, cells obtained from the PLN were re-stimulated *in vitro* for 5 days with irradiated (3300 rads) syngeneic mouse spleen cells that had been pre-treated with the monomeric form of the peptide (40 $\mu$ g/ml) for 2h at 37°C. The cell mixtures were maintained in 5-10ml of Click's medium (Click et al., 1972) supplemented with 10% fetal calf serum (FCS) and 50 $\mu$ M 2-mercaptoethanol, then washed three times with RPMI 1640 medium, 10% FCS. The CTL activity was determined by <sup>51</sup>Cr-release assay (Platsoucas & Good, 1981) against syngeneic target cells (P815) with or without pre-treatment with the monomeric form of the peptide for 2h at 37°C.

Figure 2. CTLs from Balb/c mice immunized *in vivo* with any R15K-type peptides recognize MHC-matched target cells that were either pre-treated with peptide monomers (P815+peptide) or infected with recombinant vaccinia virus expressing the HIV IIIB envelope protein, gp160 (P815+VPE16). Immunization of mice and peptide pre-treatment of P815 target cells was carried out as in Figure 1. 5 x 10<sup>6</sup> P815 target cells were infected with 5 x 10<sup>7</sup> plaque forming units of control (VSC8) or recombinant vaccinia virus expressing HIV envelope protein (VPE16) for 18-20 hours prior to labelling with 100 $\mu$ Ci of <sup>51</sup>Cr (ICN Radiochemicals, Irvine, CA). The cytotoxic activity was determined by <sup>51</sup>Cr-release assay (Platsoucas & Good, 1981).

Figure 3. CTLs primed by *in vivo* immunization with either the monomeric form or the lipid-tailed micelle form of the HIV V3-loop R15K peptide are CD8-positive. Re-stimulated PLN cells (as in FIGURE 6) were treated with complement along (+C) or with either anti-CD4 monoclonal antibody (clone GK-1.5) plus C (+ anti

CD4-C) or anti-CD8 monoclonal antibody (clone 53-6.72) plus C (+anti CD8+C) as in (Platsoucas & Good, 1981). Resulting cells were then tested for their capacity to lyse MHC-matched target cells that were either pre-treated with the monomeric form of the peptide (P815+peptide) or infected with recombinant vaccinia virus expressing HIV IIIB envelope protein gp160 (P815+VPE16).

Figure 4. CTL activity of spleen cells from <sup>W</sup>mice immunized with R15K/R15K + Th. 6-8 week old Balb/c mice were immunized in hind footpad with 100µg of R15K peptide alone or 100µg of a helper T-cell peptide (T<sub>h</sub>) emulsified in CFA. At 2, 3 or 4 weeks post-immunization, both PLN and spleen cells from the mice were collected and restimulated for 5 days with irradiated syngeneic mouse spleen cells pretreated with R15K for 2 hours. A standard 4 hour <sup>51</sup>Cr-release assay was performed, as described by Sastry et al. (1992). Representative data from PLN (A) and spleen cells (B) are shown, and demonstrate that a helper T-cell peptide from the HIV env protein enhances the systemic CTL response to R15K V3 loop peptide (seq id no:1).

Figure 5. Peptide R15K inhibits infection of human T cells (MT-4) by HIV-1. MT-4 cells are human T cells that are chronically infected by the human T cell leukemia virus type 1 and undergo lytic infection with HIV-1 (Larder et al., 1989). Therefore, inhibition of HIV-1 infection of MT-4 cells will prevent cell death. In these experiments, MT-4 cells (5x10<sup>4</sup>/100µl) were preincubated in triplicate wells of 96 well microtiter plates with various concentration of the R15K peptide for 15 minutes at 37°C, and challenged with HIV-1. Controls included cells alone and cells infected with HIV-1, but without added peptide. Seven days after infection, the total number of viable cells was determined using the MTT dye reduction assay of Larder et al. (1989). The line marked with no virus shows effect of various concentrations of peptide on the survival of MT-4 cells in the absence of HIV-1 infection. The line marked with

"v1rus" shows protective effect of peptide R15K (seq id no:1).

Figure 6. Peptide R15K inhibits HIV-1 infection of MT-4 cells as measured by a reverse transcriptase assay. In these experiments MT-4 cells were infected as in Figure 5, but instead of measuring the cytopathic effect, the amount of reverse transcriptase (RT) activity in the culture medium was determined seven days after infection by the method of Popovic et al. (1984).

Figure 7. Peptide R15K inhibits HIV-1 infection of human T cell lines CEM and H9. The CEM and H9 cells ( $1 \times 10^5$  cells/well) in separate experiments were infected with HIV-1 in triplicate wells of a 48 well plate in the presence or absence of various concentrations of peptide R15K. The experimental protocol was as described in Figure 5. Seven days after infection, reverse transcriptase (RT) activity in the culture supernatant was measured by the method of Popovic et al. (1984).

Figure 8. V-3 loop peptides inhibit HIV-1 infection of primary human T cells. Peripheral blood mononuclear cells (PBMCs) from healthy HIV-1 seronegative donors were separated by Ficoll-hypaque centrifugation. E-rosetting was used for the preparation of T cell enriched lymphocyte population, as described by Mannhalter et al. (1986). T-cells ( $1 \times 10^6$ /ml) were incubated in triplicate wells of 96 well microtiter plates with medium alone or control unrelated peptide (corresponding to a sequence in c-mos protooncogene product) or various V3 loop peptides at  $1 \mu\text{g/ml}$  concentration. Other peptides are: H13N, V3-loop peptide from HIV-1mn strain (seq id no:7); T13Q, V3-loop peptide from V3-loop of HIV-1rf strain (seq id no:6); R8K, a 8 amino acid peptide from V3-loop of HIV-1 IIIB strain (seq id no:5); N24G, a 24 amino acid peptide for HIV-1 IIIB V3-loop (seq id no:3); and R15K, a 15 amino acid peptide for HIV-1 IIIB V3-loop (seq id no:1). After 9 days, reverse transcriptase

activity in culture supernatant was determined according to the method of Popovic et al. (1984).

Figure 9. Serum stability of peptide R15K: The R15K peptide (seq id no:1) was incubated at 37°C with fetal bovine serum at 100 µg/ml concentration for 24 hours. At different time intervals aliquots of peptide/serum mixture corresponding to 1 µg/ml final concentration were added to MT-4 cells in a 96 well microtiter plate. After 15 minutes of incubation with peptide, MT-4 cells were infected with HIV-1 as described in Figure 5. Seven days after infection, the total number of viable cells was determined by the MTT-dye reduction assay (Larder et al., 1989). The controls included cells incubated in medium alone with and without virus infection and cells infected with virus and incubated with R15K peptide that was not pre-incubated in fetal calf serum.

Figure 10. Peptide E13V (seq id no:4) from the amino terminal domain of gp120 inhibits HIV-1 infection of primary human T-cells. Experimental details are same as in Figure 8 except that in this experiment, different concentrations of peptide E13V was used in place of R15K.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

AIDS is a highly infectious and fatal disease caused by a group of closely related retroviruses commonly known as HIV. There are currently no vaccines to prevent HIV infection, and no effective therapeutic agents to arrest progression once infection has been initiated. The present invention addresses both of these current failings through the use of peptide technology in the development of formulations for anti-viral vaccination and treatment.

Initially, the present inventors developed a rapid method for the identification of peptides which have the ability to induce a specific CTL response *in vivo*. This method is broadly applicable to both screening and vaccination, and is relevant to the development of preventative vaccines, not only for AIDS, but also for a wide variety of other diseases. These methods of CTL induction were then refined, by the addition of T helper cell-inducing peptides, leading to particularly effective compositions and methods for producing systemic, long-lived and high level CTL activity in response to peptide immunization.

In addition to vaccine formulations, the inventors have also identified peptide sequences which function to effectively inhibit the entry of HIV into human target cells. It is envisioned that these peptides, which arrest viral infection, will form the basis of effective anti-HIV therapeutics which halt viral spread and disease progression in HIV-infected patients.

The use of peptides, for example, instead of proteins, in clinical embodiments is generally preferred for various reasons. These include the low cost and relative ease of large scale preparation, and the reliability of the product. Also their biological properties are preferable, such as the ease with which peptides can penetrate tissues, and their low immunogenicity. Prior to clinical use, peptides may be stabilized by the addition of groups to the N- or C-termini, for example, by acylation or amidation. The formulation of the peptides of the present invention into various polymers is discussed in detail below.

One aspect of the present invention is a novel and rapid method for screening candidate compounds, such as peptides or multimers thereof, to identify appropriate CTL-inducing active compounds for use, for example, in CTL vaccines. In preferred screening procedures, candidate peptides or peptide multimers are assayed for *in vivo* CTL-induction by injecting the test substance

into an experimental animal, recovering T cells from the lymph nodes and measuring the activity (priming) of such CTLs. Most preferably immunization would involve a single intradermal injection of the test peptide in complete Freund's adjuvant (CFA) into an appropriate site, such as the hind-foot pad of a mouse, recovering lymph node T cells, preferably from the draining popliteal lymph nodes (PLN), and determining their CTL activity by assaying the T cell-mediated lysis of MHC-matched target cells decorated with the candidate peptide or expressing the parent protein. This procedure allows the results to be obtained only 8-10 days after immunization.

Employment of such a screening procedure will result in the identification of T cell active peptides, whether they be peptides that induce substantially only a CTL response, or also induce an antibody response (i.e., B cell reactive). A plurality of strains of mice that vary in their histocompatibility genes may be used for these screenings. Peptides that have a broad response in the various MHC genotypes should be selected for further study in primates, and finally humans. Exemplary assay procedures are found hereinafter.

The synthesis of peptides can be achieved using an automated peptide synthesizer. For example, peptides can be synthesized using the Merrifield solid phase method (Merrifield, 1963) either on a modified Vaga 250 automatic peptide synthesizer or by the "Bag" method, as described by Houghton et al. (1985). Following the synthetic reactions, the completed peptides are then recovered. In the above two methods this involves the removal of the T-BOC blocking groups and hydrolysis of the peptide from the resin using hydrofluoric acid (HF) treatment at 0°C for 1 hour. The various organic by-products can then be removed, for example by extraction with ether, and the peptides extracted from the supporting resin with a reagent such as 25% acetic acid.

In the rapid screening method for the identification of appropriate CTL-inducing candidate compounds, the inventors have found that the intradermal immunization of a mouse can be advantageously employed. Intradermal immunization is believed to be particularly effective as it serves to efficiently activate the cell mediated limb of the immune system. The inventors have found that even more advantageous aspects of this immunization protocol are (i) that a single injection of the candidate will usually be sufficient to achieve detectable CTL activation, and (ii) that the sensitivity of the assay is such that the use of immunization modifiers will generally not be required in order to achieve CTL priming.

Accordingly, in preferred screening embodiments, mice are immunized by intradermal injection into the hind foot-pad with an appropriate composition, such as 100 $\mu$ g of the test peptide in a 1:1 emulsion with complete freund's adjuvant (CFA). After a period of time sufficient to induce a CTL response, such as 10 days, the draining popliteal lymph nodes (PLN) are surgically removed and the cells were separated by mild homogenization. The PLN cells thus obtained, typically between 10-50 x 10<sup>6</sup> cells/lymph node/mouse, are maintained in 5-10 ml of a solution such as Click's medium, 10% FCS, 50 $\mu$ M 2-mercaptoethanol, and re-stimulated for 5 days, *in vitro*, with irradiated (3300 rads) syngeneic mouse spleen cells pre-treated with the candidate peptide. A syngeneic cell expressing the parent protein from which the peptide is derived, such as the HIV gp160 protein, would be an appropriate target cell. However, the inventors contemplate that peptide-decorated spleen cells which can be easily generated, for example by pre-treating the cells with the peptide monomer at a concentration of approximately 40  $\mu$ g/ml for 2 hours at 37°C, may be preferred for use in accordance with the present invention.

The re-stimulated effector cells are then washed, for

example with RPMI 1640 medium, 10% FCS, resuspended at an appropriate cell density in the region of  $5 \times 10^6$ /ml, and assayed for CTL activity. A suitable method of assaying for CTL activity is to determine the release of a non-metabolizable radiolabeled substance from specific target cells which have been pre-loaded with the substance. Measuring the release of  $^{51}\text{Cr}$  from peptide-treated syngeneic cells, as described by Platsoucas & Good (1981), is considered by the inventors to be a particularly suitable method.

To generate peptide-expressing target cells for use in the CTL assay, appropriate syngeneic cells, such as P815 cells, are first loaded with radioactive compound such as chromium. This can be achieved by incubating the cells for 2 hours at  $37^\circ\text{C}$  with  $250 \mu\text{Ci}$  of  $^{51}\text{Cr}$ . The cells are then washed three times and incubated for an additional 2 hours at  $37^\circ\text{C}$  with the monomeric form of the test peptide at a concentration of approximately  $40 \mu\text{g}/\text{ml}$ . The cells are then washed twice with a solution such as RPMI medium, 10% FCS, and then resuspended at an appropriate concentration, for example in the region of  $5 \times 10^4$  cells/ml. The target cells thus generated can then be employed in the CTL assay, for example by mixing them with the candidate effector cells in U-bottom 96 well-microtiter plates such that different effector to target cells (E:T) ratios are achieved.

Alternatively, as discussed above, target cells expressing the parent protein from which the test peptide was derived can be generated and used in the CTL assay. In this method, syngeneic cells are infected for 18-20 hours with an appropriate amount, such as  $5 \times 10^7$  plaque forming units, of recombinant vaccinia virus containing the gene for the parent protein which is to be expressed. As a control, syngeneic cells may be infected with recombinant vaccinia virus which carries the gene for an immunologically unrelated protein. At this stage one may wish to confirm the presence of the required protein in the target cells,



but not in the control cells, for example by western blotting both infected cell types with specific antibodies. Target cells created in this manner can then be labelled with the radioactive compound, in this case 100  $\mu$ Ci of  $^{51}\text{Cr}$ , and used in the chromium release CTL assay (Platsoucas & Good, 1981), as described above.

In certain embodiments where the candidate peptides to be screened are derived from the HIV protein gp160, the recombinant vaccinia virus system can be used to prepare cells which specifically express this protein. For use in this regard, the inventors have found the control (VSC8) and HIV env-expressing (VPE16) recombinant vaccinia viruses obtained through Dr. Bernard Moss via the AIDS Research and Reference Reagent Program, (Division of AIDS, NIAID, NIH) to be of particular use. To confirm the presence of the gp160 protein in such VPE16-infected P815 target cells, the inventors found it convenient to employ HIV antibody-positive human sera in the western blotting experiments. In using the VPE16-infected P815 target cells in CTL assays, the inventors determined that it was particularly advantageous to employ  $5 \times 10^4$  target cells/ml and  $1 \times 10^6$  candidate CTL effector cells/ml, mixed so as to obtain an initial E:T ratio of 20:1.

In CTL assays conducted using either of the above target cells, the percentage specific  $^{51}\text{Cr}$ -release can be calculated as:

$$100 \times \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}$$

The maximum release can be determined by measuring the radioactivity released into the supernatant from target cells in which complete lysis has been experimentally induced, such as by using a 5% solution of the non-ionic detergent Triton X-100 to disrupt the membranes of the cells. Spontaneous release can be

determined by measuring the radioactivity released into the supernatant from target cells incubated without added effector cells. It is contemplated that valid experiments will have a value for spontaneous target lysis which is between 15-20% of the maximum lysis observed.

Prior to assaying the PLN cells for possible CTL activity, one may desire to first deplete certain cell populations, such as CD4<sup>+</sup> or CD8<sup>+</sup>, from the mixture of cells. In this regard, re-stimulated PLN cells can be treated with either anti-CD4 monoclonal antibody (clone GK-1.5) plus complement (C) (+ anti CD4+C), or anti-CD8 monoclonal antibody (clone 53-6.72) plus C (+anti CD4+C), as described by Platsoucas & Good (1981). It may also be desirable to treat certain PLN cells with complement alone (+C), to act as a comparison. Cells treated in any of these ways can then tested for their capacity to lyse MHC-matched target cells that have been pre-treated with the monomeric form of the peptide or infected with recombinant vaccinia virus expressing the parent protein from which the test peptide was derived.

Where one desires to identify CTL reactive compounds that do not exhibit an antibody generating capability, peptides identified as being T cell active may be screened to identify those that lack B cell stimulatory activity. Such peptides are proposed to be particularly useful in the preparation of vaccines for the treatment or prevention of viral diseases such as AIDS where an antibody response may in fact enhance infectivity of the causative agent. Identification of such peptides is accomplished by injecting a candidate into an immunocompetent animal (e.g., mice) to identify those peptides that fail to generate a significant antibody response to the native protein to whose sequence the peptides correspond in part. For example, in the case of HIV, one would desire to test for the production of antibodies having reactivity against gp120, gp41 or core

proteins, etc.

Of course, as mentioned above, the invention also concerns the identification of compounds capable of eliciting both a T cell response and a B cell response, in that this latter group will serve to induce protective antibody-based immunity in the immunized host.

CTL active compositions of the present invention prime T cells in a way that, when the infecting virus appears at a later date, memory T cells are activated to result in a cell-mediated immune response that destroys target cells that have the corresponding target protein or a portion thereof on their cell surfaces, and thereby the virus.

The inventors further optimized the above-described method for the generation of peptide-induced CTL responses against peptide immunogens. Particular objectives were to increase specific CTL production by lymphoid tissue more distant from the site of injection, such as in the spleen, and to improve the longevity of the response. It was determined that physically mixing peptides containing T helper epitope sequences with CTL-inducing peptides enhanced CTL responses in mice, both following multiple subcutaneous (sc) injections and single intradermal (id) injections in the footpad. In addition, the high level CTL response in the spleen was maintained for up to eight weeks after one id injection. An example of a suitable T helper peptide is C19A, having the sequence CRIKQIINMWQGVGKAMYA (seq id no:2), which was found to effective in monomer, lipid tailed, and di-sulphide polymer forms.

The mechanism of enhancing a peptide-induced CTL response in vivo by peptides that induce T helper cells is not known. However, it is likely that the enhancing peptide, because of its ability to induce T helper cells, will result in increasing the

levels of necessary cytokines that assist in the clonal expansion and dissemination of specific CTLs. Regardless of the underlying mechanism, it is envisioned that the use of mixtures of helper T cell and CTL-inducing peptides in immunization or vaccine formulations will greatly improve the capability of generating a systemic CTL response with a relatively long half life, compared to immunization with the CTL-inducing peptide(s) alone.

With regard to HIV, it is proposed that particularly useful peptides against which to generate a CTL responses will be those peptides which have sequences derived from the viral genes *gag*, *pol*, *nef* and *env* genes (Table 1), with *env* genes being particularly preferred. Even more preferably, peptides with sequences derived from the V3 loop of HIV gp120, such as those listed in Table 2, may be employed as CTL-inducing peptides. It is envisioned that T helper cell-inducing epitopes for use in the present invention will generally have having an amphipathicity value of from about plus 10 to about plus 20.

Peptides and peptide combinations considered to be suitable as vaccines or components thereof may be tested in experimental animal models, such as mice, rats, rabbits, guinea pigs, goats, Rhesus monkeys, chimpanzees, and the like. Such tests will likely lead to the production of successful vaccines that induce cell-mediated immunity for protection against a variety of infectious agents.

Further aspects of this invention concern the identification and formulation of synthetic peptides as therapeutic agents for use against HIV, such that these chemically defined synthetic peptides will protect human cells from HIV-1 infection.

The V3 loop region of gp120 is known to be essential for HIV-1 infection of cells (Travis et al., 1991; Freed and Riser, 1987; Freed et al., 1991). Anti-V3 loop antibodies have been

shown to inhibit HIV-1 cellular infection without interfering with gp120 binding to its cellular receptor, CD4 (Linsley et al., 1988; Javaherian et al. 1989). The V3 loop region is also known to be a target for a trypsin related protease on the host cell surface (Murakami et al., 1991). It has been speculated that after gp120 binds to CD4, the V3 loop is cleaved by a cell surface protease leading to a conformational change in the gp120/gp41 protein complex (McCune et al., 1988). Such a change may then expose the fusogenic domain in the transmembrane protein gp41, resulting in the fusion of the viral particle membrane with the cell membrane. The inventors therefore reasoned that V3 loop peptides may prevent infection of cells by inhibiting the cleavage of the gp 120 V3 loop.

Recent studies in this area have lead to the generation of conflicting results. Koito et al. (1989) reported that a synthetic peptide, corresponding to the major HIV-1 neutralizing epitope from the V3 loop of gp120, inhibits syncytium formation between the HIV-1 infected CCRF-CEM and uninfected Molt-4 cells in a dose-dependent manner. In these studies the 36 amino acid peptide (residues 303-338), CTRPNNNTRKSIRIQRGPGRAFTIGKIGNMRQAHC, representing the entire V3 loop, was found to inhibit syncytium formation by 30-60% at a concentration of 100  $\mu$ M (approximately 300  $\mu$ g/ml). However, a 24 amino acid peptide (residues 308-331, NNTRKSIRIQRGPGRAFTIGKIG) representing the middle portion of the V3 loop was less efficient and was required at 300  $\mu$ M concentration (approximately 720  $\mu$ g/ml) to achieve the same level of inhibition of syncytium formation. Neither of these peptides were tested for their capacity to inhibit HIV infection of cells.

In contrast, De Rossi et al. (1991) recently reported that synthetic peptides of 24 amino acids in length from V3 loop regions of different HIV-1 isolates actually enhance HIV-1 infection of cells through a CD4-dependent mechanism. In these experiments synthetic peptides at concentrations ranging between

0.62-20  $\mu$ M (approximately 1.5 to 48  $\mu$ g/ml) were employed and one human T cell line (Molt-3) was tested.

Despite the above evidence to the contrary, the present  
5 inventors proceeded to test the ability of V3 loop synthetic  
peptides to inhibit HIV infection. Synthetic peptides of varying  
length (8-24 amino acids) selected from the V3 loop of gp120 were  
determined to be surprising effective at inhibiting HIV infection  
of T cells, using both cultured human T cells such as H9, CEM and  
10 MT-4, and freshly prepared primary human T cells. These peptides  
include R15K, having the sequence RIQRGPGRAFVTIGK (seq id no:1),  
and N24G, having the sequence NNTRKSIRIQRGPGRAFVTIGKIG (seq id  
no:3). The peptides termed D23, D24, D25, D26, D30, D35, D38,  
D39 and D40, the sequences of which are shown in Table 11A, were  
15 also found to inhibit HIV cellular infection.

An important discovery of this invention is the finding that  
peptides with sequences from a variety of different HIV isolates  
have the ability to inhibit HIV-1 IIIB infection of cells. These  
20 include H13N (HIGPGRAFYTTKN, seq id no:7) from HIV-1mn, and T13Q  
(TKGPGRVIYATGQ, seq id no:6) from HIV-1rf, along with the  
peptides detailed in Table 11A. Importantly, it is contemplated  
that peptides with sequences which correspond to V3 loop  
sequences other viral strains will also be useful in inhibiting  
25 infection by the homologous, or other related, HIV strains. As  
such, it is contemplated that peptides such as D27, D28, D29,  
D31, D32, D33, D34 AND D37 (Table 11B) may still be found to be  
useful as infection-inhibiting peptides.

30 The inventors have previously shown the peptide  
EQLWVTVYYGVPV (E13V, seq id no:4), from the amino-terminus of  
gp120 (residues 39-51) to be capable of inducing anti-HIV  
T helper cell responses in mice (Sastry & Arlinghaus, 1991).  
Antibodies against this peptide were found to be capable of  
35 binding denatured, but not native, gp120. On analyzing the

ability of peptide E13V (seq id no:4) to interfere with HIV entry into cells, and thus to inhibit infection, the inventors found it, also, to be an effective infection-inhibitor peptide.

5           The infection-inhibitor peptides of the present invention, such as R15K (seq id no:1) and E13V (seq id no:4), are envisioned to be ideal candidates for inclusion in therapeutic formulations that will be effective in preventing infection of human cells by HIV-1. Of relevance to human therapy is the finding that R15K,  
10           for example, retained full strength inhibitory activity after incubation in fetal calf serum for up to 4 hours. Since such formulations will be completely synthetic and defined, these reagents will be both economical and safe. Also, the V3 loop peptides as a mixture will provide the added advantage of being a  
15           immuno-therapeutic reagent because of the capacity of inducing HIV-1 specific CTL responses that can effectively kill all virus infected cells while preventing fresh infection of cells.

20           Thus, the present invention ultimately involves the preparation of both vaccines and therapeutic formulations including synthetic peptides. It is envisioned that the vaccines will include a peptide(s) bearing CTL-inducing epitopes, and ideally, will also include a peptide(s) bearing T helper cell-inducing epitopes. The therapeutic formulations may also, of  
25           course, include such sequences. However, peptides incorporating an HIV infection-inhibiting sequence are particularly preferred for use as components of such a therapeutic composition.

30           With regard to vaccination-type peptides of the present invention, it is contemplated that peptide multimers may be particular advantageous in the preparation of CTL-inducing vaccines in accordance herewith. Preferred multimers are formed of surfactant-like micelles and polymers. In addition to the amino-terminal lysyl residue, the spacer peptide can contain one  
35           to about five additional residues. Substantially any amino acid

residue can be utilized so long as it does not interfere with the T cell immunizing capacity of an aqueous composition containing the multimer or with the capacity of the di-amide reaction product to form surfactant-like micelles in an aqueous composition. One to about three glycyl residues per spacer peptide are preferred.

The before-described peptide and the amino-terminal lysyl residue-containing peptide spacer are peptide-bonded together, and can thus be viewed as a composite polypeptide. The useful diamide is thus a reaction product of the alpha- and epsilon-amino groups of the amino-terminal lysyl residue and two moles per composite polypeptide of the C<sub>12</sub>-C<sub>18</sub> fatty acid. The composite polypeptide can thus be prepared as a single sequence and amidified before or after removal from the resin, where solid phase synthesis is used, by conventional techniques.

The phrase "surfactant-like micelle" is used herein to emphasize that, in an aqueous composition, the di-amidolysyl composite polypeptide appears to form micelles similar to those formed by surfactants and to distinguish such multimers from submicroscopic structural units of protoplasm built up from polymeric molecules that are also sometimes referred to as micelles. The word "micelle" is also sometimes used herein, and when so used has the same meaning as surfactant-like micelle.

Another multimer form of a previously described peptide is a polymer having a plurality of peptide repeating units. In this case, a peptide containing two terminal cysteines as part of its natural sequence can be selected and synthesized. A peptide lacking such cysteines can be modified by the addition of one or two extra cysteines to the N- and C-terminal ends, respectively. The presence of two cysteines per peptide permits polymerization of the subunit peptide by air oxidation to form oxidized cysteine(cystine)-linked polymers and/or cyclic peptides. Such



multimers enhance immune recognition of the peptide without the need of a carrier.

5 The lysine-terminated spacer peptide can contain one to about five amino acid residues in addition to the lysyl residue, and the one or two added terminal cysteine residues are not included in counting the length of a peptide of the present invention. A peptide containing terminal cysteine residues is referred to as a di-cysteine-terminated peptide or more simply, a  
10 di-Cys peptide. Details for preparing polymers containing di-Cys peptide repeating units are provided hereinafter.

Aqueous compositions (inocula) of the present invention comprise an immunologically effective amount of a CTL-inducing, T helper cell-inducing, or HIV infection-inhibiting peptide or peptides, whether in multimeric form or not, dissolved or dispersed in a pharmaceutically acceptable aqueous medium. Such compositions are also referred to as inocula.

20 The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

25 The preparation of an aqueous composition that contains an immunizing molecule such as a peptide or multimer, or an infection-inhibiting component, such as a peptide, an as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in,  
30 or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

A peptide or peptide multimer can be formulated into a composition in a neutral or salt form. Pharmaceutically  
35 acceptable salts, include the acid addition salts (formed with

the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine, and the like.

Upon formulation, vaccines will be administered in a manner compatible with the dosage formulation, and in such amount as is immunologically effective. As used herein, "immunologically effective amount" means an amount of composition is used that contains an amount of a peptide, peptides, or peptide multimers sufficient to induce an effective CTL response in the host animal (mammal) such as by the induction of specifically targeted cytotoxic T cells. In preferred embodiments, it is contemplated that the immunologically effective vaccine composition will also include an amount of a peptide, peptides, or peptide multimers effective to induce T helper cell activity and to thus enhance the CTL effect. The presence of such cytotoxic T cells is assayed as discussed hereinafter.

In the context of a vaccine, the quantity of peptide(s) and volume of composition to be administered depends on the host animal to be immunized, the capacity of the host animal's immune system to activate T cells, and the degree of protection desired. Precise amounts of active peptide multimer required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosage ranges are of the order of about 10 micrograms ( $\mu\text{g}$ ) to about 500 milligrams (mg), preferably about  $50\mu\text{g}$  to about 1mg, and more preferably about  $100\mu\text{g}$  of active ingredient peptide multimer per individual. A minimal volume of a composition required to disperse the immunizing amount of peptide multimer is typically utilized.

Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed in one or two week intervals by a subsequent injection or other administration.

5

A composition can also include an adjuvant as part of the excipient. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) for use in laboratory host mammals are well known in the art, and are used illustratively herein. Pharmaceutically acceptable adjuvants such as alum can also be used.

10

The following examples are included to demonstrate preferred embodiments of the invention. It will be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art will, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

15

20

25

**EXAMPLE 1 - Preparation of Peptides, Peptide Polymers and Peptide Micelles**

Synthetic peptides of 7 to about 30 amino acid residues in length were prepared corresponding to the selected conserved domains of the core and gp160 (gp120 and gp41) molecules using the solid-phase technique of Merrifield (1963) using a modified Vega 250 automated peptide synthesizer or by the "bag" method described in Houghten (1985). In either case, removal of the t-butyloxycarbonyl (t-BOC) amino acid blocking groups and the

30

35



TABLE 3  
AMINO ACID SEQUENCE OF SYNTHETIC PEPTIDES

	PEPTIDE # ENVELOPE	AMINO ACID SEQUENCE <sup>1</sup>	LOCATION IN HIV <sup>2</sup>
5	103	39EQLWVTVYYGVPV <sup>51</sup>	GP160-CR-1
	104	45VYYGVPVWKEA <sup>55</sup>	GP160-CR-1
	105	48GVPVWKEATTLC <sup>61</sup>	GP160-CR-1
10	106	72AHK VWATHACV <sup>82</sup>	GP160-CR-1
	107	81CVPTNPVPQEVV <sup>92</sup>	GP160-CR-1
	108	92VLENTENFNM <sup>102</sup>	GP160-CR-1
	109	105NNMVEQMHEDI <sup>116</sup>	GP160-CR-1
	110	109EQMHEDIISLWDQ <sup>121</sup>	GP160-CR-1
15	111	118LWDQSLKPCVKLT <sup>130</sup>	GP160-CR-1
	112	121SLKPCVKLTPLC <sup>133</sup>	GP160-CR-1
	113	204SVITQACSKVSFE <sup>216</sup>	GP160-CR-2
	114	215FEPIPIHYCAFPGF <sup>228</sup>	GP160-CR-2
	115	236KKFNGTGPCTN <sup>246</sup>	GP160-CR-2
20	116	240GTGPCTNVSTVQC <sup>252</sup>	GP160-CR-2
	117	250VQCTHGIRPVVSTQ <sup>263</sup>	GP160-CR-2
	61	586YLRDQQLLGIWGC <sup>598</sup>	GP160-CR-5
	63	519FLGFLGAAGSTMGAASL-	
		TLTVQANQ <sup>543</sup>	GP160-CR-5
25	65	417CRIKQIINMWQGVGKAMYA <sup>435</sup>	GP160-CR-3
	67	417CRIKQIINMWQGVGKAM-	
		YAPPIGGQIRC <sup>444</sup>	GP160-CR-3

1. The N- and C-terminal amino acid residues of each peptide are numbered as to their position in the gp160 amino acid residue sequence according to Modrow *et al.* *Virology*, 61:570 (1987). A dash (-) indicates that the sequence continues on the next line.

2. CR = Conserved Region

Two types of high molecular weight (multimeric) forms of the peptides listed in Table 3 were prepared. The principal form of multimer was a di-cysteine (di-Cys terminated) polymer in which a plurality of peptides were linked end-to-end by disulfide bonds.

5 These di-cysteine polymers were produced by adding cysteine residues to the termini of each peptide during synthesis. The di-cysteine-terminated (di-Cys) peptides were then dissolved (10mg/ml) in ammonium bicarbonate (0.1M) at room temperature (~25°C) and stirred for about 16 hours to effect oxidation of the  
10 sulfhydryl groups to produce polymer forms of the peptides. The peptide solution was freeze-dried and analyzed by HPLC to confirm the presence of polymer forms of the peptide.

15 The second type of high molecular weight form produced was a surfactant-like micelle formed by linkage of an amino-terminal lysine-containing spacer peptide (Lys-Gly-Gly-) to the peptide sequence to form a composite polypeptide, and then coupling a C<sub>12</sub>-C<sub>18</sub> fatty acid, such as palmitic acid, to both the alpha and epsilon amino groups, as in Hopp (1984). The C<sub>12</sub>-C<sub>18</sub> fatty  
20 acid-containing peptides produced are then extracted in 95% acetic acid and utilized to form large micelles in the aqueous composition that exhibit increased immunogenicity relative to the peptides.

25 Di-Cys polymer multimers of all of the peptides listed in Table 3 were prepared. Aqueous peptide micelle multimers have been prepared of peptides designated 61, 63, 65 and 67, and are designated as peptides 62, 64, 66 and 68, respectively. Peptides designated 103 through 117 were utilized only in their di-Cys  
30 polymer multimeric forms.

35 The high molecular weight, multimeric forms produced correspond to multiple copies of specific regions of gp120 and gp41 in HIV. For ease of designation, the multimer forms will be designated by the peptide number from which it is composed - that

is, peptide 61 refers to the di-Cys multimeric (polymeric) form of peptide 61 and peptide 66 refers to the aqueous micelle form of peptide 65, whereas peptide 103-117 refers to a polymeric multimer.

5

Peptides 65 and 66 correspond to the region of gp120 that binds to the cell surface T4 receptor. Peptides 61 and 62 correspond to a region near the amino-terminal portion of gp41 that represents a major immunodominant epitope seen by AIDS patients' sera.

10

## **EXAMPLE 2 - Anti-Peptide Antibody Response**

Aqueous compositions of the multimers; i.e., the di-Cys peptide polymers and micelles produced in EXAMPLE 1 were assayed for their ability, or lack of ability to elicit an anti-peptide antibody response in BALB/c mice, an immunocompetent mouse strain.

15  
20  
25

Groups of BALB/C mice (6-8-week-old females, 3 to 5 mice/group, Charles River Laboratories) were immunized by subcutaneous (s.c.) or intraperitoneal (i.p.) injection of a peptide multimer (100µg/injection) in complete Freund's adjuvant (CFA) (1:1 ratio). Booster injections (50µg of peptide multimer) in incomplete Freund's adjuvant (IFA) (1:1) were given at 6 and 10 weeks after the initial immunization. Each mouse was bled from its retro-orbital plexus at two-week intervals and the serum was pooled for individual mice in each group.

30

An ELISA assay was performed on each serum to detect the presence of anti-peptide antibodies utilizing peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) as the second antibody. Preliminary results for peptides 61-68 are shown in Table 4, whereas further refined

35

results for peptides 61, 63, 65, 67 and 103-117 are shown in Table 5. It was found that the high molecular weight forms of peptides 65, 66, 67, 68, 105 to 110, 112, 114, 115 and 117 elicited high antibody titers, whereas peptides 61, 62, 63, 64, 103, 104, 111, 113 and 116 produced very low to negligible amounts of anti-peptide antibodies. Similar results were obtained for antibody responses in B6C3 F1 mice (Charles River Laboratories), another immunocompetent strain.

Some of the sera were further assayed for antibody response (reactivity) with native gp160, and the results, shown in Table 6, demonstrate that these peptides do not represent B cell epitopes since there was no immunoreaction with native gp160.



**TABLE 4**  
**ANTIBODY RESPONSE OF VARIOUS PEPTIDES IN BALB/C MICE**

5	Peptide 1	Pre- Immune	ELISA Titer in Bleed 1							
			1	2	3	4	5	6	7	8
10	61	1:40	1:400	1:100	1:100	a:100	1:100	1:400	1:200	1:400
	62	1:20	1:100	1:100	1:100	1:100	1:100	1:200	1:100	1:100
	63	1:40	1:80	1:20	1:40	1:320	1:80	1:80	1:320	1:5120
	64	1:20	1:40	1:40	1:00	1:40	1:40	1:40	1:40	1:40
15	65	1:40	1:80	1:800	1:1x10 <sup>4</sup>	1:5x10 <sup>4</sup>	1:2x10 <sup>4</sup>	1:2x10 <sup>5</sup>	1:2x10 <sup>5</sup>	1:2x10 <sup>5</sup>
	66	1:40	1:160	1:6x10 <sup>3</sup>	1:1x10 <sup>5</sup>	1:1x10 <sup>5</sup>	1:1x10 <sup>5</sup>	1:2x10 <sup>4</sup>	1:5x10 <sup>4</sup>	1:5x10 <sup>5</sup>
	67	1:40	1:160	1:3x10 <sup>3</sup>	1:2x10 <sup>4</sup>	1:2x10 <sup>4</sup>	1:1x10 <sup>5</sup>	1:8x10 <sup>5</sup>	1:1x10 <sup>5</sup>	1:2x10 <sup>5</sup>
	68	1:80	1:1600	1:1x10 <sup>4</sup>	1:1x10 <sup>4</sup>	1:1x10 <sup>5</sup>	1:1x10 <sup>5</sup>	1:4x10 <sup>5</sup>	1:4x10 <sup>5</sup>	1:4x10 <sup>5</sup>

**TABLE 5**  
**ANTIBODY RESPONSE OF VARIOUS PEPTIDES IN BALB/C MICE**

	PEPTIDE	ELISA TITER
5	#62 AA 586-598	1:400
	#63 AA 519-543	1:5120
	#65 AA 417-435	1:2x10 <sup>5</sup>
	#67 AA 417-444	1:82x10 <sup>5</sup>
10	#103 AA 39-51	1:640
	#104 AA 45-55	1:2000
	#105 AA 48-61	1:5000
	#106 AA 72-82	1:4x10 <sup>5</sup>
	#107 AA 81-92	1:1x10 <sup>5</sup>
15	#108 AA 92-102	1:1x10 <sup>5</sup>
	#109 AA 105-116	1:8x10 <sup>5</sup>
	#110 AA 109-121	1:6x10 <sup>5</sup>
	#111 AA 118-130	1:80
	#112 AA 121-133	1:1x10 <sup>5</sup>
20	#113 AA 204-216	1:640
	#114 AA 215-228	1:1x10 <sup>6</sup>
	#115 AA 236-246	1:4x10 <sup>5</sup>
	#116 AA 240-252	1:640
	#117 AA 250-263	1:8x10 <sup>6</sup>

**TABLE 6**  
**T AND B CELL RESPONSES IN MICE TO HIV**  
**ENVELOPE GP160 DERIVED SYNTHETIC PEPTIDE IMMUNOGENS**

Peptide Immunogen	In Vitro Proliferation of PLN Cells from*				Antipeptide Antibody Reactivity to**	
	B <sub>6</sub> C <sub>3</sub> F <sub>1</sub>		A SWxBalb/c F <sub>1</sub>			
	Analogous Peptide	GP 160	Analogous Peptide	GP 160	Analogous Peptide	GP 160
61	++	+	++	++	-	-
63	++	++	++	++	±	-
65	++	+	++++	++	++	-
67	++	-	++++	++	+++	-
103	+	+	+++	+	-	-
104	++++	++	+++	+	±	-
105	++++	+++	+	-	±	-
106	+++	+	++++	+	++	-
107	++	++	+	+	+	-
108	+	+	+	-	+	-
109	++	±	±	+	+++	-
110	++	-	++	+	++++	-
111	+	++	±	-	-	-
112	+	+	+	+	+	-
113	++	+	++	+	-	-
114	++	-	++	+	++++	-
115	++	+	▲ND	ND	++	-
116	++	-	ND	ND	-	-
117	+++	-	ND	ND	++++	-

\* cpm values are corrected and categorized according to unrelated antigen response *in vitro*.

\*\* Antibody raised in Balb/C mice, reactivity measured by ELISA and categorized according to the end point.

▲ Not determined.

### EXAMPLE 3 - T Cell Responses

The high molecular weight, multimeric di-Cys peptide polymeric forms of the peptides described in EXAMPLE 1 were assayed for their elicitation of a T cell proliferative response as in Millich et al. (1985).

Mice (3 or 5 mice/group) were injected in the right hind footpad with a 1:1 mixture of peptide polymer (100µg/injection) and CFA . Peptides 61, 63, 65 and 67 were injected into B6C3 F1 mice (H-2<sup>kxb</sup>, Charles River Laboratories) and A.SWxBALB/C F1 mice (H-2<sup>sxd</sup>, Jackson Labs, Bar Harbor, ME). Draining popliteal lymph node (PLN) cells were harvested after ten (10) days, and cultured (2x10<sup>5</sup> cells/well) in 96-well microtiter plates in 0.2 ml of Click's medium (Click et al., 1972) containing various concentrations of synthetic peptide, gp160, an unrelated proteinaceous material or medium alone, for 96 hours at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. During the final 16-18 hours of culturing, <sup>3</sup>H-thymidine (<sup>3</sup>H-TdR) (1µCi/well, 6-7 Ci/mmol, ICN Radiochemicals) was added. The cells were harvested onto filter strips and <sup>3</sup>H-TdR incorporation was monitored.

Results from such studies were expressed as a stimulation index (SI) representing the fold increase in radioactivity counts in the presence of antigen compared to background values where no antigen was added. The SI values with the different peptides were compared to that obtained with tuberculin purified protein derivative (PPD) as a positive control antigen.

The peptide-specific <sup>3</sup>H-TdR incorporation for T cell responses (delta cpm) in mice with the differing major histocompatibility (MHC) haplotypes, B6C3 F1 (C57B1/6xC<sup>3</sup>H/HcJ) and (A.SWxBALB/c) F1, were determined for all of the synthetic peptides. The <sup>3</sup>H-TdR incorporation values represent the difference between the radioactivity values obtained in wells

containing antigen and in control wells without added antigen. The non-specific proliferation of PLN cells was determined by including an unrelated peptide in the assays, shown as a horizontal bar for each peptide.

5 All of the assayed peptides were found to exhibit good T cell proliferative responses in B6C3 F1 mice, whereas all of the assayed peptides, except peptides 105, 107, 109 and 111, exhibited good T cell proliferative responses in A-SWxBALB/c F1 mice.

10 It was demonstrated by the results above and those described in EXAMPLE 2 that peptides 61, 63, 103, 104 and 113 do not stimulate anti-peptide antibody production but are very good immunogens, in their disulfide (di-Cys) polymeric form, for eliciting a strong T cell response directed against both the corresponding peptide and the native HIV envelope protein gp160.

15 T cell proliferation measured by  $^3\text{H}$ -TdR incorporation, was also similarly assayed as a function of the T cell antigen concentration, using various amounts of native gp120 or gp160 as one control, and PPD as another control. PLN from B6C3 F1 mice were used in these studies.

#### 20 25 **EXAMPLE 4 - Induction of HIV-Specific Cytotoxic T Lymphocytes**

Groups of 3 to 5 syngeneic female mice (6 to 8 weeks of age) are immunized by intradermal injection in an appropriate site with an aqueous composition containing an immunizing (CTL-stimulating) amount of peptide either as monomer or as the before-discussed multimers in CFA (1:1). Ten (10) days after immunization, draining PLN cells and spleen lymphocytes are obtained and restimulated *in vitro* by culturing for six (6) days with irradiated syngeneic normal spleen cells that were pre-

treated with the same synthetic peptide as immunogen.

The presence of cytotoxic T lymphocytes (CTL) is determined by a 4-hour  $^{51}\text{Cr}$  release assay as follows. The PLN cells are maintained for five days at  $37^\circ\text{C}$  in Clicks medium containing 10% fetal calf serum (FCS) together with irradiated syngeneic normal spleen cells that were pre-treated with the appropriate test peptide. These cells are designated as the effector cells, and express H-2<sup>d</sup> MHC class I antigen.

Target cells (phytohemagglutinin-stimulated (PHA) blasts of syngeneic mouse spleen cells or P815 mouse cells expressing a corresponding HIV protein) are washed three times with serum-free RPMI 1640 medium and then admixed, contacted and maintained (incubated) at  $37^\circ\text{C}$  for about 1.5 to about 2 hours together with  $250\mu\text{Ci}$  of sodium chromate (specific activity 200-400 Ci/g of  $^{51}\text{Cr}$ , New England Nuclear, Boston, MA). The target cells are subsequently washed with RPMI 1640 medium containing 10% FCS, and resuspended in RPMI 1640 with 10% FCS and different concentrations of peptide. These cells are then washed 3 times with RPMI containing 10% FCS and resuspended at  $5 \times 10^4$  cells/ml. A  $100\mu\text{l}$  aliquot of each cell suspension is added to a well of a 96-well-U-bottom microtiter plate.

A  $100\mu\text{l}$  aliquot of the appropriate effector cell suspension ( $5 \times 10^6$  cells/ml) is added to each well and a twofold serial dilution made to obtain different effector-to-target cell (E:T) ratios. Control wells receive 0.1ml of RPMI medium with 10% FCS alone in the absence of effector cells to obtain a value for spontaneous  $^{51}\text{Cr}$  release, and receive 0.1ml of 5% Triton X-100 detergent to obtain a value for maximum  $^{51}\text{Cr}$  release.

The plates are incubated at  $37^\circ\text{C}$  for about 4 hours, following which  $100\mu\text{l}$  of supernatant from each well is monitored in a gamma counter to determine  $^{51}\text{Cr}$  release. The percent

cytotoxicity is calculated as

$$\frac{(\text{Effector Cell-Stimulated Release}) - (\text{Spontaneous Release})}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100$$

**EXAMPLE 5 - Rapid assay of HIV-specific CTLs Induced by an Immunodominant peptide.**

A peptide with the sequence RIQRGPGRAFVTIGK, herein referred to as R15K (seq id no:1), from the HIV gp160 immunodominant V3 loop has previously been identified as a CTL immunodominant epitope in H-2<sup>d</sup> mice (Takahashi et al., 1988). In the original studies, CTLs induced *in vivo* by infecting Balb/c mice with recombinant vaccinia virus expressing HIV env proteins, were shown to lyse syngeneic target cells pre-incubated with R15K. Unfortunately, to date, studies in which mice were immunized with free R15K peptide have been reported to be unsuccessful in inducing CTLs (Berzofsky, 1991).

Accordingly, the present inventors sought to examine the induction of CD8<sup>+</sup> HIV R15K-specific CTLs in 6-8 week old Balb/c mice by employing differing sites of inoculation, differing forms of the peptide, and recovering the effector cells from different tissue origins. It was observed that draining popliteal lymph nodes (PLN) of mice immunized in the hind-foot pad with 100μg of the R15K peptide (seq id no:1), in a 1:1 emulsion with CFA, were the best source of CTL effectors against irradiated (3300 rads) syngeneic target cells preincubated with monomeric R15K (40μg/ml for 2h at 37°C). This has particular importance and physiological significance because human lymph nodes have been described as the primary site of HIV replication (Kaneshima et al., 1991; Fauci 1991). An important aspect of this immunization

protocol is that CTLs could be recovered by mild homogenization from PLN surgically removed after only 10 days.

To determine the optimal form of the peptide for consistent induction of peptide-specific CTLs *in vivo*, the R15K peptide (seq id no:1) was prepared in three different configurations:

a) linear monomer, b) disulfide-linked polymer formed by oxidation of cysteine residues added at both the N- and C-termini and c) micelles formed by conjugating the peptide to a

dipalmityl-lysine-glycine-glycine at the N-terminus (Hopp, 1984; Sastry & Arlinghaus, 1991). It was found that a single immunization of Balb/c mice in the hind-foot pad with any of the above R15K forms in CFA consistently resulted in generation of CTLs which specifically lysed MHC-matched target cells (P815, H-2<sup>d</sup>) pre-incubated with the peptide. Such responses were observed in 8 of 12 mice immunized with the monomeric form, in 13 of 13 mice immunized with the micelle form and 6 of 8 mice immunized with the disulfide polymer form of the peptide. Lysis of MHC-matched target cells without peptide pre-treatment (P815) was not observed. Representative results are shown in Figure 1.

The CTLs induced by all three forms of the peptide also specifically lysed P815 cells infected with a recombinant vaccinia virus expressing HIV gp160 (VPE16), but not cells infected with a control vaccinia virus (P815+VSC8) (Figure 2). Western blotting with HIV antibody-positive human sera confirmed the presence of gp160 protein in VPE16-infected, but not in VSC8-infected P815 target cells. The peptide-induced CTLs in Balb/c mice were H-2 restricted. They lysed only peptide pre-treated H-2<sup>d</sup> target cells (P815) but not peptide-treated 3A9 target cell, which are expressing the H-2K haplotype (Table 7).

Representative results with CTLs generated in mice immunized with the micelle form of R15K (seq id no:1) peptide are shown in Table 7. Similar results were obtained with CTLs induced by



injection of the monomeric and polymeric forms of the R15K peptide.

5 Experiments were performed to determine whether the virus-specific CTLs were CD8<sup>+</sup> or CD4<sup>+</sup>. Treatment of the CTL effectors from mice immunized with monomeric peptide with anti-CD8 monoclonal antibodies (MAbs) and rabbit complement abolished the cytotoxicity against peptide treated, or env expressing, targets (Figure 3A). In contrast, pre-treatment of effector cells with  
10 anti-CD4 MAbs plus complement, or complement alone, had no significant effect. Similar results were also obtained with CTLs generated in mice immunized with the peptide in micelle (Figure 3B) and polymeric forms. The induction of CD8<sup>+</sup> CTLs by R15K (seq id no:1) is consistent with the use of P815 target  
15 cells which express MHC class I, but not class II, gene products (Maryanski et al., 1985). MHC class I-restriction is commonly observed with CD8<sup>+</sup> effector CTLs.

20 Since the CTL epitope studied in the present investigation is in the middle of an immunodominant B-cell active region of the HIV gp120, the B-cell activity of the R15K peptide was investigated. Either as monomer or lipid tailed micelle or after conjugation to KLH, this peptide failed to induce in mice a measurable titer of anti-peptide antibody. However, the mice  
25 immunized with peptide conjugated to KLH did make antibodies against KLH, showing that the mice are immunocompetent. Sera from mice immunized in the foot pad were also tested, when it was observed that no anti-peptide antibodies were formed.

TABLE 7

Peptide and Target Cell Specificity of CTL's induced  
in Balb/C Mice by the HIV env V3-Loop R15K Peptide (seq id no:1)

Target	% Specific Lysis at Various E:T <sup>a</sup> Ratios		
	100:1	50:1	25:1
Cells			
P815 <sup>b</sup>	7.2	0	0
P815 + A84 <sup>c</sup>	76.7	67.2	65.9
P815 + B106 <sup>d</sup>	4.2	0.6	0
P815 + B105 <sup>e</sup>	6.7	0	0
3A9 <sup>f</sup>	7.0	0.6	0
3A9+A84 <sup>g</sup>	10.0	8.0	0

- a= Effector to target cell ratio  
b= MHC-matched target cells (H-2<sup>d</sup>)  
c= H-2<sup>d</sup> cells pre-treated with HIV env V3-loop R15K peptide  
d= H-2<sup>d</sup> cells pre-treated with a Influenza virus peptide  
e= H-2<sup>d</sup> cells pre-treated with a Sendai virus peptide  
f= MHC-mis-matched target cells (H-2<sup>k</sup>)  
g= H-2<sup>k</sup> target cells pre-treated with HIV env peptide V3-loop

Despite the results presented immediately above, the location of R15K in a variable region (V3-loop) of HIV gp160 could be viewed as a reason for not selecting this peptide as a potential vaccine candidate. However, a comparison of gp160 amino acid sequences from 245 different HIV isolates has shown that as little as five different consensus sequences can be defined on a serological basis among all the viral isolates (LaRosa et al., 1990). Therefore, the inventors propose that a cocktail of CTL-inducing peptides from V3-loop regions encompassing all the principal HIV groups (which may be five or less) may be sufficient for generating CTLs specific for cells expressing gp120 from most if not all HIV strains. Such a mixture would then serve as a prototype vaccine for evaluation for prevention of HIV infection of humans.

**EXAMPLE 6 - Enhancement of Virus-Specific CTL Responses by  
T helper cell-inducing Peptides.**

5       The present example concerns a method for enhancing the  
systemic distribution, level of activity, and longevity of virus-  
specific CTLs induced in response to CTL epitope-bearing  
peptides. This method involves the addition of a separate and  
distinct class of peptides to the immunization mixture that  
10       possess T helper cell-inducing activity as a means to enhance the  
CTL-inducing capacity of a given peptide immunogen.

15       The inventors method for inducing CTL responses against  
peptide immunogens, as described in Example 5, results in rapid  
CTL induction in the proximal lymph node (near the site of  
injection). However, it was noted that distant lymphoid tissue  
(i.e. spleen) accumulated a lower level of antigen-specific CTLs.  
The inventors next reasoned that the further induction of  
T helper cell activity may be advantageous in improving the  
dissemination of specific CTLs. As the method described above  
20       allows screening of CTL-inducing peptides without the need to  
include T helper epitope sequences, the inventors tested whether  
HIV T-cell helper peptides physically mixed with the HIV-specific  
CTL-inducing peptide (RIQRGPGRAFVTIGK, R15K, seq id no:1) would  
25       enhance the observed CTL response in mice following multiple  
subcutaneous (sc) injections. The T helper peptide selected for  
these studies was CRIKQIINMWQGVGKAMYA, C19A, (seq id no:2), which  
had been previously demonstrated to have T helper cell-inducing  
activity.

30       Balb/c mice were injected sc three times at bi-weekly  
intervals with R15K (seq id no:1) peptide mixed with the T helper  
peptide prepared in one of three forms: monomer, lipid tailed and  
di-sulphide polymer. One week after the last injection, spleen  
35       cells were obtained, restimulated and tested for CTL activity.  
This experiment was successful and significant CTL activity was



TABLE 8

CTL Activity of Spleen Cells From Balb/c Mice  
Immunized Subcutaneously (sc) with a Mixture of R15K (seq no:1)  
and the T-Helper Cell peptide C19A (seq id no:2)

		%specific Lysis at various E:T <sup>a</sup> ratios			
Immunogen	Target cells	200:1	100:1	50:1	25:1
R15K+122 <sup>b</sup>	p815 <sup>e</sup>	22.9	12.5	7.9	5.1
	p815+R15K <sup>f</sup>	53.6	47.4	34.0	25.2
	p815+122 <sup>g</sup>	10.6	5.4	3.8	1.3
R15K+65 <sup>c</sup>	p815	8.4	13.4	7.5	4.4
	p815+R15K	48.4	27.9	25.3	12.9
	p815+122	6.8	9.9	0	0
R15K+66 <sup>d</sup>	p815	27.4	16.0	16.0	10.5
	p815+R15K	58.8	37.4	33.8	20.7
	p815+122	16.3	7.0	4.1	2.2

a= Effector to target cell ratios

b= T=Helper cell peptide in its monomeric form mixed with R15K

c= T=Helper cell peptide in its polymeric form mixed with R15K

d= T=Helper cell peptide in its lipid-tailed form mixed with R15K

e= MHC-matched target cells (P815, H-2<sup>d</sup>)

f= P815 target cells pre-incubated with R15K

g= P815 target cells pre-incubated with monomeric form of T-helper cell peptide.

Mice were immunized subcutaneously with the peptide mixture emulsified in complete Freund's adjuvant (1:1) at bi-weekly intervals for six weeks (total 3 injections). Spleens were harvested one week after the last injection and restimulated for 5 days, as described by Sastry et al., 1992. Peptide 122 is a T-helper cell stimulating peptide that has the sequence CRIKQIINMWQGVGKAMYA, also herein referred to as C19A (seq id no:2); R15K is a CTL-inducing peptide that has the sequence RIQRGPGRAFTIGK (seq id no:1). Both sequences originated from the gp120 sequence of HIV-1 (Sastry et al., 1991; 1992).

TABLE 9

CTL Activity of Spleen Cells from Balb/c Mice Immunized  
Subcutaneously (sc) with a Mixture of R15K (seq id no:1) and  
the T-Helper Cell peptide C19A (seq id no:2)

		%specific Lysis at various E:T <sup>a</sup> ratios				
# of	Injections	Target cells	100:1	50:1	25:1	12.5:1
1		p815 <sup>c</sup>	2.9	4.7	3.3	3.3
		p815+R15K <sup>d</sup>	11.0	7.7	3.6	0.1
		p815+122 <sup>e</sup>	9.4	3.8	2.2	1.6
2		p815	5.5	3.8	1.6	1.8
		p815+R15K	13.1	11.0	5.0	0.8
		p815+122	3.0	4.3	2.8	1.8
3		p815	6.6	3.6	4.3	4.3
		p815+R15K	13.7	4.3	5.6	0.5
		p815+122	10.5	5.2	4.9	6.8

a= Effector to target cell ratios

b= Mice were immunized either once, twice or three times with a mixture of R15K and the monomeric form of T-helper cell peptide

c= MHC-matched target cells (P815, H-s<sup>d</sup>)

d= P815 target cells pre-incubated with R15K

e= P815 target cells preincubated with the monomeric form of T-helper cell peptide

Mice were immunized subcutaneously with the peptide mixture emulsified in CFA (1:1) at two week intervals. One week after each injection, spleen cells were harvested and restimulated as described by Sastry et la., 1992.

To date the R15K peptide by itself has not been shown by any other group to be capable of inducing a CTL response. But the present inventors, as disclosed herein, have demonstrated CTL induction in the popliteal lymph node within 7 days by a single id immunization of Balb/c mice with R15K alone (Figure 4A). It was therefore reasoned that a mixture consisting of R15K and the T helper peptide when injected once by the id route might be sufficient to achieve systemic spread and longevity of HIV-specific CTL response.

A number of mice were immunized with a single id injection in the footpads with either the CTL peptide (R15K) alone or in a mixture with the T helper peptide C19A (seq id no:2). The results (Figure 4) showed that mice immunized with the mixture had substantially higher CTL responses in the spleen than mice receiving just the R15K peptide alone (Figure 4B). In addition, the high level of CTL response in the spleen was maintained for up to eight weeks after a single id injection, whilst the helper T-cell peptide (C19A, seq id no:2) injected along lacked CTL-inducing activity.

Lasarte et al. (1992) recently reported that multiple intraperitoneal injections of mixtures of an R15K-bearing CTL epitope and a helper T-cell epitope, KQUIINMWQEVGKAMYA, in mice induced a low level HIV-specific CTL response after three weeks. However, in these experiments, the CTL peptide by itself was not capable of inducing HIV-specific CTLs even after multiple injections. Therefore, the role of T helper peptide in these studies is not clear. On the other hand, the studies described above show that the CTL-peptide possesses the CTL-inducing capacity and the role of T helper peptide is to disseminate and enhance that inherent CTL response of the CTL-peptide. These studies therefore indicate that in order to achieve an efficient, systemic and long lasting cell-mediated immunity, the candidate vaccine preparation should ideally include both T helper and CTL

peptides. Another important aspect of this invention is that such a mixture given once intradermally is sufficient to induce a long lasting systemic antigen-specific CTL response.

5  
**EXAMPLE 7 - Rapid Induction of Influenza Virus- and Sendai Virus-Specific CTLs**

10 The protocol developed for the induction of HIV-specific CTLs, as described above in Example 5, was believed to be generally applicable to the identification, selection and assay of any peptide with unknown epitope specificity, for its ability to prime CTLs *in vivo*. Accordingly, the *in vivo* peptide-  
15 induction of CTLs specific for influenza virus was examined. Deres et al., (1989) had previously shown that a synthetic peptide R<sup>-</sup>, TYQRTALVTG (aa 147-158), corresponding to a portion of the nucleoprotein of influenza virus, could prime influenza virus-specific CTLs in mice *in vivo* only when covalently linked through the N-terminus to tripalmitoyl-S-glycerylcysteinyl-seryl-  
20 serine (P<sub>3</sub>CSS). However, using the protocol described above, specific CD8<sup>+</sup> CTLs, that lysed target cells pre-treated with this peptide, could be induced *in vivo* by immunization with the free synthetic peptide (Table 10).

25 Employing this immunization protocol, an *in vivo* CTL response was also successfully generated against the unmodified free synthetic peptide, HGEFAPGNYPALWSYA, which represents the immunodominant CTL epitope from the nucleoprotein of Sendai virus  
30 (B105: NP 321-336).

35 Thus, this method was indeed found to be useful in systems other than those related to the HIV virus. Furthermore, it is believed that this rapid screening method will have medical utility for developing candidate vaccines and therapeutics for various infectious diseases.



TABLE 10

In Vivo Priming of Peptide Specific CTLs in Balb/C Mice with a Free Synthetic Peptide (B106) from Influenza Virus Nucleoprotein

Treatment To Effector Cells	Target	% Specific Lysis at Various E:T <sup>a</sup> Ratios				
		160:1	80:1	40:1	20:1	
No Treatment	Cells					
	P815 <sup>b</sup>	5.7	0	0	2.5	
No Treatment	P815+B106 <sup>c</sup>	83.9	76.6	57.1	47.3	
No Treatment	P815+B105 <sup>d</sup>	11.0	26.0	19.3	16.0	
+ Complement	P815+B106	83.3	73.6	58.2	39.7	
+ anti-CD4 +C	P815+B106	67.2	59.3	40.7	25.8	
+ anti-CD8 +C	P815+B106	2.5	0	0	0.4	
+ anti-CD4	P815+B106	64.8	67.9	44.7	24.8	
+ anti-CD8	P815+B106	53.6	43.3	23.7	5.8	
No Treatment	3A9 <sup>e</sup>	0	0	ND	ND	
No Treatment	3A9+B106 <sup>f</sup>	0	0	0	0	

ND= Not Done

a= E:T = Effector to target cell ratio

b= MHC-matched target cells (H-2<sup>d</sup>)

c= H-2<sup>d</sup> target cells pre-treated with Influenza virus peptide

d= H-2<sup>d</sup> target cells pre-treated with Sendai virus peptide

e= MHC-mis-matched target cells (H-2<sup>k</sup>)

f= H-2<sup>k</sup> target cells pre-treated with Influenza virus peptide

**EXAMPLE 8 - Inhibition of HIV-1 Infection and Syncytia Formation  
by Human Cells by Synthetic Peptides from gp120.**

The present example describes the identification and use of  
5 synthetic peptides, derived from gp120, to protect human cells  
against HIV-1 infection, and to inhibit syncytia formation. MT-4  
cells are human T cells that are chronically infected by the  
human T cell leukemia virus type 1 and undergo lytic infection  
with HIV-1 (Larder et al., 1989). Therefore, inhibition of HIV-1  
10 infection of MT-4 cells will prevent cell death.

The ability of gp120 derived synthetic peptides to inhibit  
HIV infection of cells was investigated. Evidence was obtained  
to demonstrate that synthetic peptides of varying length (8-24)  
15 amino acids) selected from the V3 loop of gp120 inhibited HIV  
infection of both cultured human T cells (Figures 5, 6 & 7) as  
well as freshly prepared primary human T cells (Figure 8).

It was observed that both the 24 amino acid peptide N24G  
20 (aa 308-311, NNTRKSIRIQRGPGRAFTIGKIG, seq id no:3) and the 15  
amino acid peptide R15K (aa 315-329, RIQRGPGRAFTIGK, seq id  
no:1), with sequences derived from the V3 loop of HIV-1 IIIB,  
inhibited HIV-1 infection of primary human T cells by 92% at  
1  $\mu$ g/ml (approximately 0.4-0.6  $\mu$ M) concentration (Figure 8). An  
25 8 amino acid shorter form of the V3 loop peptide, R8K (aa 322-  
329, RAFVTIGK, seq id no:5) also showed 66% inhibition of HIV-1  
infection of primary human T cells at 1  $\mu$ g/ml (approximately  
1.25  $\mu$ M) concentration (Figure 8). Synthetic peptides from V3  
loop regions of heterologous isolates, HIV-1mn (T13Q,  
30 TKGPGRVIYATGQ, seq id no:6) and HIV-1rf (H13N, HIGPGRAFYTTKN, seq  
id no:7), also showed significant inhibition (>60%), each at 1  
 $\mu$ g/ml (approximately 0.78  $\mu$ M) concentration, of cells by the IIIB  
strain (Figure 8).

35 A variety of other peptides with sequences derived from the

V3 loops of other HIV-1 strains were also found to exhibit significant activity with respect to the inhibition of HIV-1 IIIB infection of human cells. As detailed in Table 11A, these peptides include D23, D24, D25, D26, D30, D35, D38, D39, D40 and D44, which reflect a variety of strains such as mn, rf, wmj-3, sc, z6, eli, mn (y-l) and mn (y-p). However, it is important to note that this assay was confined specifically to inhibiting the infection of the heterologous strain HIV-1 IIIB. Therefore peptides, such as those in Table 11B, which did not show activity in this specific assay may still have utility as infection-inhibiting sequences to combat the variety of HIV strains known to be present in the infected human population.

**TABLE 11A**  
**EFFECT OF V3 LOOP PEPTIDES ON HIV-1 IIIB INFECTION OF HUMAN CELLS**

	PEPTIDE NO.	STRAIN	SEQUENCE	RT ACTIVITY (CPM)	% RT INHIBI- TION
5	D23 (24aa)	MN	YNKRKRIHIGPGRAFYTTKNNIGC	94	98.8
	D24 (15aa)	MN	RIHIGPGRAFYTTKN	5000	22.1
	D25 (15aa)	WMJ-3	SLSIGPGRAPRTREI	4342	42.4
	D26 (24aa)	RF	NNTRKSITKGPGRVVIYATGQIIGD	2093	72.2
10	D30 (15aa)	SC	SIHIGPGRAFYATGD	3443	54.3
	D35 (15aa)	Z62	STPIGLGQALYTTTRG	5242	30.3
	D38 (15aa)	ELI	RTPTGLGQSLYTTTRS	5823	33.7
	D39 (15aa)	MN(Y-L)	RIHIGPGARFLTTKN	1978	73.8
	D40 (15aa)	MN(Y-F)	RIHIGPGRAFFTCKN	2805	62.8
15	D44 (15aa) R15K	IIIB	RIQRGPGRAFTIGK	2021	73.2
	Uninfected cells			558	-

**TABLE 11B**  
**EFFECT OF V3 LOOP PEPTIDES ON HIV-1 IIIB INFECTION OF HUMAN CELLS**

	PEPTIDE NO.	STRAIN	SEQUENCE	RT ACTIVITY (CPM)	% RT INHIBITION
	D27 (15aa)	NY-5	GIAIGPGRTLYAREK	7657	0
	D28 (15aa)	RF	SITKGPGRVVIYATGQ	7634	0
	D29 (15aa)	CDC4	RVTLGPGRVWYTTGE	13009	0
30	D31 (15aa)	Z3	SIRIGPGKVFTAKGG	11877	0
	D32 (15aa)	SF2	SIYIGPGRAFHTTGR	8002	0
	D33 (15aa)	MAL	GIHFGPGQALYTTGI	8959	0
	D34 (15aa)	Z321	SISIGPGRAFFATTD	11142	0
	D37 (15aa)	JY1	STPIGLGQALYTTTRI	7027	0
35	No Peptide			7528	0
	Uninfected cells			558	-

The capacity of peptide E13V, EQLWVTVYYGVPV (seq id no:4), from the amino-terminal portion of gp120 to inhibit HIV-1 infection of primary human T cells was also determined. It was observed that this peptide, at as low as 1 ng/ml concentration (approximately 0.77 nM), inhibited HIV-1 infection by 90% (Figure 10).

Further studies were conducted to determine the effect of V3 loop synthetic peptides from different HIV-1 strains on syncytium formation. For these studies HeLa CD4 cells were infected with recombinant vaccinia viruses expressing the envelope protein gp160 of HIV-1 IIIB, mn or rf strains, at an m.o.i. of 100, in the presence and absence of V3 loop peptides from respective the HIV-1 stains (i.e., R15K, H13N and T13Q). At 18 hours post infection, cells were observed under microscope, using a magnification of 100, for syncytia.

In these studies, no syncytia were observed in cells incubated with the synthetic peptides before infection with the respective recombinant vaccinia viruses. These results clearly demonstrate the capacity of the V3 loop peptides to inhibit cell to cell spread of HIV-1.

Studies were conducted to examine the stability of the R15K peptide from HIV-1 IIIB isolate in serum. In these studies the R15K (seq id no:1) peptide was incubated in fetal calf serum at 37°C and at different time intervals during the incubation aliquots at a final concentration of 1 µg/ml (approximately 0.6 µM) were tested for inhibition of HIV-1 infection of a human T cell line (MT-4 cells). The results are shown in Figure 9. The R15K peptide retained its full strength inhibitory activity for up to 4 hours and 50% of control untreated peptide inhibitory activity was retained even after 24 hours incubation at 37°C in fetal calf serum.

It is envisioned that stability examinations will be conducted on any synthetic peptide, or mixtures thereof, identified for potential clinical use. Such tests will include, for example, pre-incubation in human serum and plasma; treatment with various proteases; and also temperature- and pH-stability analyses. If found to be necessary, the stability of the synthetic peptides may be enhanced by any one of a variety of methods such as, for example, employing d-amino acids in place of l-amino acids for peptide synthesis; using blocking groups like t-boc and the like; or encapsulating the peptides within liposomes. The bio-availability of select mixtures of peptides may also be determined by injecting radio-labelled peptides into mice and rhesus monkeys and subsequently analyzing their tissue distribution.

In addition to the inventors' previous demonstration that the R15K peptide can induce CTL responses (see above, Example 5), they also show this peptide and its analogues from other HIV-1 isolates, to efficiently protect human T cells from HIV-1 infection. This activity was demonstrated using cultured cells such as H9, CEM and MT-4 cells, and freshly prepared human T cells. These results indicate that the V3 loop peptides can act in two separate ways: 1) to induce HIV-1 specific cytotoxic T lymphocytes that specifically kill cells expressing HIV-1 gp120; and 2) to prevent infection of normal cells by infectious virus. Thus, these V3 loop peptides have utility not only for vaccines but also as therapeutic reagents to prevent HIV-1 infection in humans or reduce the spread of virus infection in HIV-infected individuals.

**EXAMPLE 9 - Induction of HIV-specific T cell Responses in Monkeys  
on Immunization with a Synthetic Peptide Cocktail**

5 The present example describes the successful induction of  
HIV-specific T cell responses in rhesus monkeys with a mixture of  
eight synthetic peptides, seven of which were derived from  
conserved regions of the HIV-1 envelope protein. These results,  
demonstrating the induction of HIV-1 specific T cell responses in  
a non-human primate model, constitute an important step towards  
10 identifying and formulating a synthetic peptide-based vaccine  
that can induce a broad based cell-mediated immunity for  
protecting humans against HIV infection.

15 In the present studies, three rhesus monkeys (#7, #23 and  
#283C) were immunized subcutaneously with 1 ml (0.1 ml/site) of a  
mixture of eight synthetic peptides (300  $\mu$ g of each peptide in  
sterile water) emulsified in complete Freund's adjuvant at 1:1  
ratio. These eight peptides, termed 61, 63, 104, 105, 111, 113,  
116 and R15K (Table 12), had previously been identified as gp160-  
20 specific T cell active synthetic peptides. At 3 and 7 weeks  
after the primary immunization, two booster injections of the  
peptide mixture (150  $\mu$ g of each peptide in sterile water)  
emulsified in incomplete Freund's adjuvant were given to each  
monkey. Monkey #7 was terminated after 34 weeks, for health  
25 reasons unrelated to the study, while the remaining two monkeys  
(#283C and #23) received one additional booster injection at 25  
weeks.

TABLE 12  
AMINO ACID SEQUENCES OF IMMUNIZING PEPTIDES

5	peptide 61	aa 586-598	YLRDQQLLGIWGC
	peptide 63	aa 519-543	FLGFLGAAGSTMGAASLTTLTVQARC
	peptide 104	aa 45-55	VYYGVPVWKEA
	peptide 105	aa 48-61	GVPVWKEATTLFC
	peptide 111	aa 118-130	LWDQSLKPCVKLT
10	peptide 113	aa 204-216	SVITQACSKVSFE
	peptide 116	aa 240-252	GTGPCTNVSTVQC
	peptide R15K	aa 315-329	RIQRGPGRFVTIGK

The numbering of amino-and carboxy terminal amino acids of each peptide are according to the sequence reported by Modrow et al., 1987.

At two week intervals following the first immunization of each animal, 15 ml of whole heparinized blood was collected by venous puncture. The peripheral blood mononuclear cells (PBMC) were separated by standard ficoll-hypaque centrifugation and employed in proliferation assays. The PBMCs were monitored every two weeks for a period of 32 weeks for proliferative responses against individual peptides and recombinant gp160.

It was found that PBMCs from all three rhesus monkeys showed good proliferative responses with peptides 104 (aa 45-55), 111 (aa 118-130) and 63 (aa 519-543); while weak responses were observed with peptides 113 (aa 204-216) and 116 (aa 240-252) (Table 13). Two of the three rhesus monkey-derived PBMC preparations also showed good proliferative responses with peptide 61 (aa 586-598) (Table 13). A significant response was not detected in any of the monkeys with peptides 105 (aa 48-61) and R15K (aa 315-329). PBMCs from all three monkeys showed



significantly high proliferative responses with recombinant gp160, the HIV-1 envelope protein precursor, for the entire period of the experiment.

TABLE 13  
T-cell Proliferation Responses

Peptide	Rhesus Monkey		
	#7	#23	#283C
104 (aa 45-55)	4+	2+	4+
111 (aa 118-130)	3+	2+	3+
63 (aa 519-543)	2+	4+	3+
113 (aa 204-216)	1+	2+	2+
116 (aa 240-252)	1+	3+	2+
61 (aa 586-598)	-	3+	2+
R15K (aa 315-329)	-	-	-
GP160 (aa 1-863)	6+	4+	4+

(-), no detectable response; (1+), poor response; (2+), good response; (3+), better response; (4+, 6+), best response.

These results demonstrate that mixtures of synthetic peptides from HIV env gene product can prime gp160-specific T cell responses in rhesus monkeys. Because of their ability to induce specific T-cell responses both in mice and rhesus monkeys, these HIV env peptides are proposed to be useful as components of vaccines to prevent HIV infection in humans.

\*

\*

\*

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## REFERENCES

The following references are hereby incorporated by reference for the subject matter as specified in the specification and to the extent that they disclose, teach, enable or provide a basis for various aspects of the present invention.

Ahearne et al., III International Conference on AIDS, held in Washington, D.C., June 1-5, 1987, abstract # M.10.3, page 8

Aichele, P. Hengartner, H. Zinkernagel, R.M. & Schulz, M.J., Exp. Med. 171:1815-1820 (1990)

Barnes, Science, 236:255 (1987)

Berzofsky et al., Nature, 334:706-708 (1988)

Berzofsky, J.A., FASEB J., 5:2412-2418 (1991,

Bodansky et al., Peptide Synthesis, John Wiley and Sons, Second Edition, (1976)

Buller et al., Nature, 328:77 (1987)

Cease et al., Proc. Natl. Acad. Sci. USA, 84:4249-4253 (1987)

Click et al., Cellular Immunol. 3:264-276 (1972)

Coates et al., Nature, 326:549 (1987)

Culmann et al., Eur. J. Immunol., 19:2383 (1989)

Dadaglio et al., J. Immunol., 147:2302 (1991)

De Lisi & Berzofsky, Proc. Natl. Acad. Sci. USA, 82:7048 (1985)

Deres, K. Schild, H. Wiesmuller, K-H. Jung, G. & Rammensee, H-G., Nature 342:561-564 (1989)

De Rossi et al., Virology 184:187-196 (1991)

Doherty et al., Adv. Cancer Res., 42:1 (1985)

Earl et al., Science, 234:728 (1986)

Fathman, Ann. Rev. Immunol., 5:477 (1987)

Fauci, A., AIDS Update 4:103 (1991)

Freed & Risser, J. Virol. 61:2852-2856, (1987)

Freed et al., Proc. Natl. Acad. Sci. USA 87:4650-4654 (1990)

5 Gao et al., J. Immunol. 147:3268-3273 (1991)

Hart et al., Proc. Natl. Acad. Sci. USA 88:9448-9452 (1991)

10 Ho et al., J. Virol., 61:2024 (1987)

Hopp & Woods, J. Mol. Biol. 23:807 (1982)

Hopp, T.P., Mol. Immunol. 21:13-16 (1984)

15 Hosmalin et al., Proc. Natl. Acad. Sci., 87:2344 (1990)

Houghten, Proc. Natl. Acad. Sci, USA, 82:5131-5135 (1985)

20 Javaherian et al., Proc. Natl. Acad. Sci. U.S.A. 86:6768-6772  
(1989)

Johnson et al., J. Immunol., 147:1512 (1991)

25 Johnson et al., J. Exp. Med., 175:961-971 (1992)

Kaneshima, H. et al., Proc. Natl. Acad. Sci. U.S.A. 88:4523-4527  
(1991)

30 Kast, W.M. et al., Proc. Natl. Acad. Sci. U.S.A. 88:2283-2287  
(1991)

Koenig, S. et al., J. Immunol., 145:127 (1990)

Koito et al., Int. Immunol. 1:613-618 (1989)

35 Kyte & Doolittle, J. Mol. Biol., 157:105-132 (1982)

Larder et al., Science, 243:1731-1734 (1989)

40 LaRosa, G.J. et al., Science 249:932-935 (1990)

Lasarte et al., Cell. Immunol. 141:211-218 (1992)

45 Legrain et al., J. Virol., 60:1141 (1986)

Linsley et al., J. Virol. 62:3695-3702 (1988)

Livingston & Fathman, Ann. Rev. Immunol. 5:477 (1987)

50 Maddon et al., Cell, 47:333 (1986)

Maizel et al., Eur. J. Immunol. 10:509 (1980)

Mannhalter et al., J. Immunol. 136:2792-2799 (1986)

Maryanski, J.L. et al., Eur. J. Immunol. 15:1111-1117 (1985)

5 McCune et al., Cell 53:55-67 (1988)

McOmie, J.F.W., Protective Groups in Organic Chemistry, Plenum Press, New York, (1973).

10 Merrifield, J. Am. Chem. Soc. 85:2149-2154 (1963)

Michel et al., AIDS Research and Human Retrovirus, 8(4):469-478 (1992)

15 Milich and McLachlan, Science, 234:1398, (1986)

Millich et al., J. Immunol. 134:4194-4203 (1985)

Milich et al., J. Exp. Med. 164:532 (1986)

20 Mittler, R.S. & Hoffman, M.K., Science 245:1380-1382 (1989)

Modrow et al., J. Virol., 61:570-578 (1987)

25 Murakami et al., Biochem. Biophys. Acta 1079:279-284 (1991)

Naso et al., J. Virol., 45:1200 (1983)

Nixon, D.F. et al., Nature 336:484-487 (1988)

30 Platsoucas, C.D. & Good R., Proc. Natn. Acad. Sci. U.S.A. 78:  
4500-4505 (1981)

Popovic et al., Science, 224:497-500 (1984)

35 Rees, Nature, 326:343 (1987)

Robey et al., Proc. Natl. Acad. Sci., USA 83:7023 (1986)

40 Sastry, K.J. & Arlinghaus, R.B., AIDS, 5:699-707 (1991)

Sastry et al., Virology, 188:502-509 (1992)

Senyk et al., J. Exp. Med., 133:1294 (1971)

45 Sette et al., Mol. Immunol., 23:807 (1986)

Siliciano, R.F. et al., Cell, 54:561-575 (1988)

50 Takahashi, H. et al., Proc. Natn. Acad. Sci. U.S.A., 85:3105-3109 (1988)

Townsend, A.R.M. et al., Cell, 44:959-968 (1986)

Travis et al., Virology, 186:313-317 (1992)

5 Wain-Hobson et al., Cell, 40:9 (1985)

Walker et al., Science, 234:1563-1566 (1986)

Walker et al., Nature, 328:345 (1987)

10 Walker et al., Proc. Natl. Acad. Sci., 86:9514 (1989)

Weinhold, K.J. et al., J. Immunol. 142:3091-3097 (1989)

2025090-9320930